Factors affecting pigmentation quality in Atlantic salmon (*Salmo salar* L.) at elevated temperature

By

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The UTAS Animal Ethics Committee (AEC) in accordance with the “Australian code for the care and use of animals for the scientific purposes” approved the care and use of fish for this experiment (AEC approval number A0014015).

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ABSTRACT

Pigmentation quality is one of the most important criteria in Atlantic salmon (*Salmo salar*) products. The red colour is a result of pigment carotenoids such as astaxanthin (Ax) and canthaxanthin (Cx) deposited in myofibrillar proteins of white muscle tissue and the concentration of these pigment carotenoids correlate well with the red colour intensity. Sea water temperatures in Tasmanian commonly exceed 20°C for prolonged periods in summer. This is associated with reduced pigmentation quality, which is characterised by reduced red colour intensity and increased heterogeneity in pigmentation on the fillet surface of individual salmon, leading to substantial product downgrade. This phenomenon is typically concurrent with reduced feed intake rates or even the cessation of feed intake. The aim of the thesis was to improve the fundamental understanding of factors and mechanisms that reduce pigmentation quality in salmon exposed to elevated temperature in order to improve pigmentation management in salmon farming.

Post-smolts that doubled initial weights at an elevated temperature (19.5°C) showed increased concentrations of Ax in white muscle compared to fish held at a control temperature (15°C). However, in contrast to 15°C, the concentration of Ax was lower in the anterior/dorsal fillet cut (ADC), compared to the dorsal Norwegian quality cut (dNQC) at 19.5°C. Differences in the Ax concentration per unit of crude protein in white muscle between the fillet cuts at 19.5°C may have indicated differences in the affinity of myofibrillar proteins in muscle to bind Ax. A novel experimental *in vivo* carotenoid depletion model (four weeks starvation-challenge at 19.5°C) was developed. After starvation-challenge, fish showed a marked drop of redness chromaticity (a*) by image analysis on the ADC. A follow-up study confirmed the image analysis results by a marked reduction of Ax concentration in the ADC, and there was also a reduction of Ax in the dNQC when fish were starved at 20.8°C for four weeks. Fish starved for the same period of time at 15°C also showed a loss in Ax concentration in the ADC. Thus, starvation is the main factor that causes reduced pigmentation quality and elevated temperature (20.8°C) exacerbated the effect. Pigment carotenoids are potent antioxidants, but the concentration of Ax was not associated with oxidative stress (OS) in muscle of either growing or starving fish. Further, an increase in the

concentration of the antioxidant α-tocopherol in muscle did not prevent the reduction of a*-values at starvation-challenge. There was no idoxanthin (Ix), the first metabolic product of Ax, in the muscle of either growing or starving fish, which showed that metabolic conversion did not explain the differences in the concentration of Ax. Further, despite the lipophilic nature of carotenoids and interactions with dietary lipids, the dietary fatty acid (FA) composition did not affect Ax deposition at 15°C and 19.5°C in feeding salmon, and lipid fluxes in a subsequent starvation phase at 15°C and 20.8°C showed no association with the reduction in the concentration of Ax. However, a change in FAs used as energy substrate in white muscle occurred during starvation and was related to the reduction of the concentration of Ax. These changes may have indicated that a switch in lipid metabolism may have been associated with the reduction of Ax concentration. Energy homeostasis in animals cells is complex and a change in lipid metabolism is likely linked to other changes which may also affect muscle proteins. Proteolytic processes are elevated in immature salmonids at starvation and in maturing salmonids during spawning migration. During spawning migration, anadromous salmonids starve, use vast amounts of muscle protein as energy substrate and reallocate carotenoids from muscle into other tissues.

Globally rising sea water temperatures require more knowledge on the effects of elevated temperature on the biology and product quality of aquaculture species produced in the marine environment. The current research indicated that reduced pigmentation quality in salmon at elevated temperature was not associated with OS, the metabolic conversion of Ax to Ix, or interactions with lipids, respectively. However, the differences in the Ax concentration per unit crude protein at elevated temperature, and changes in energy homeostasis associated with the reduction in Ax concentration at starvation indicated that proteolytic processes could be involved. In order to further the findings of this thesis, the effects of muscle proteolytic processes on pigmentation quality in salmon fillets at elevated temperature warrants further research.

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List of abbreviations

ADC	anterior/dorsal fillet cut
ADCT	apparent digestibility coefficient
ANOVA	analysis of variance
A-Toc	α-tocopherol
Ax	astaxanthin
BW	body weight
$Carot_{total}$	total carotenoid concentration
CP	crude protein
CSI	cardio somatic indices
CT	control temperature
Cx	canthaxanthin
DHA	docosahexaenoic acid
DM	dry matter
DNPH	dinitrophenylhydrazine
dNQC	dorsal part of the Norwegian quality cut
D1	diet containing fish oil as the major oil source
D1CT	diet containing fish oil as the major oil source at control temperature (treatment)
D1ET	diet containing fish oil as the major oil source at elevated temperature (treatment)
D2	diet containing canola oil as the major oil source
D2CT	diet containing canola oil as the major oil source at control temperature (treatment)
D2ET	diet containing canola oil as the major oil source at elevated temperature (treatment)
EPA	eicosapentaenoic acid
ET	elevated temperature
FA	fatty acids
FCR	feed conversion ratio
GSH	glutathione
GSSG	glutathione disulphide
HPLC	high performance liquid chromatography
HSI	hepatosomatic indices
K	Fulton's condition factor

LC-PUFA	long chain polyunsaturated fatty acids
MDA	malondialdehyde
MUFA	monounsaturated fatty acids
OS	oxidative stress
OSI	oxidative stress index
PL	phospholipids
RAS	recirculating aquaculture system
ROS	reactive oxygen species
SEM	standard error of the mean
SFA	saturated fatty acids
SGR	specific growth rate
TAG	triglycerides
tGSH	total glutathione
TLE	total lipid extract
UPLC	ultra performance liquid chromatography
UV	ultraviolet

CHAPTER 1

General Introduction

1.1 Introduction

Salmonids have the ability to retain relatively high amounts of pigment carotenoids in white skeletal muscle tissue, which causes the typical red colouration of salmon flesh (Rørvik et al., 2010). The willingness of consumers to pay for salmon products is strongly linked to the red colour intensity (Alfnes et al., 2006) and thus, the concentrations of pigment carotenoids retained in flesh (Buttle et al., 2001). Salmon producers therefore attach high importance to pigmentation quality when salmon reach market size (3-6 kg).

Atlantic salmon are the largest and most valuable intensive aquaculture species and grown globally including in Tasmania, Australia (Jobling et al., 2010). Salmon farmers around Tasmania experience reduced pigmentation quality around the summer period, which is characterised by reduced red colour intensity and increased heterogeneity in pigmentation on the fillet surface of individual salmon, leading to substantial product downgrade. (T. Fox-Smith, pers. communication). Sea water temperatures around Tasmania commonly exceed 19°C for prolonged periods and reach over 23°C in some places (Stehfest et al., 2017). This phenomenon is concomitant with decreased feed intake or even the cessation of feed intake (T. Fox-Smith, pers. communication). This study aimed to identify factors leading to, and to determine mechanisms involved in, the reduced pigmentation quality in Atlantic salmon (*Salmo salar* L.) at elevated temperature (ET).

1.2 Carotenoids; more than expensive pigments

Carotenoids are isoprenoid pigments which are produced by bacteria, algae and plants and can emerge in various colours (Lerfall, 2016). The annual bioproduction of carotenoids in nature is estimated around 100 million tonnes, which makes carotenoids one of the largest and most widespread pigment groups (Lerfall, 2016). About 700 carotenoids are known (Esatbeyoglu and Rimbach, 2016; Lerfall, 2016), of which around 100 were identified in marine products (Lianen-Jensen, 2012). Lycopene, a C_{40} conjugated polyene chain is the basic carotenoid structure (Lerfall, 2016). Physically, the colour of carotenoids is due to a chromatophore of variable length, constituted of conjugated double-bonds, leading to various colours, and the colour changes manifest with an increasing number of conjugated double bonds (Lerfall, 2016) The natural colour of carotenoids may vary, depending on which structural component the carotenoids are further bound to. For instance, in different crustacean tissues, the colour spectrum of astaxanthin reaches from orange/red to blue and green, depending on which carotenoprotein astaxanthin is bound to (Shahidi et al., 1998). The

two main pigment carotenoids known to give salmonid flesh the red colour are astaxanthin (3,3'-dihydroxy-ß,ß-carotene-4,4'-dione; Ax) and canthaxanthin (ß,ß-carotene-4, 4'-dione; Cx) (Figure 1.1) (Buttle et al., 2001; Ingle de la Mora et al., 2006; Ytrestøyl and Bjerkeng, 2007a). Ax is predominantly found in wild salmonids (Foss et al., 1984) and accumulates via the food chain (Edwards et al., 2016). Synthetically produced Ax and Cx represent the major source in salmon farming (Higuera-Ciapara et al., 2006) and concentrations of 6-7 mg/kg flesh are considered satisfactory in harvest sized fish (Torrissen and Christiansen, 1995). However, pigment carotenoid concentrations between different fillet areas may vary (Refsgaard et al., 1998) and other factors like: total lipid concentration, carotenoid type (Ax vs Cx) (Bjerkeng, 2000) and maybe even the organisation of carotenoids into muscle proteins (Johnston et al., 2006) may affect the visualisation and red colour intensity of pigment carotenoids. This makes it difficult to set firm thresholds for the concentration of carotenoids in salmon flesh to be satisfactory for marketing.

In numerous aquaculture species, carotenoids can serve as antioxidants, hormone precursors, immune health promoters, vitamin A precursors, sex-signalling ornaments, growth promoters, and as protectors against damage induced by UV light (Christiansen et al., 1995b; Lorenz and Cysewski, 2000). Synthetic and natural Ax share the same identical optical and geometrical isomers and are distinguishable due to different isomer ratios (Edwards et al., 2016). Synthetic Ax and Cx have been thoroughly investigated by European and American health institutes and classified as safe for human consumption when not exceeding a (conservatively estimated) daily intake of 0.03 mg/kg body weight (Edwards et al., 2016; Esatbeyoglu and Rimbach, 2016). The intake of very high doses of concentrated Cx

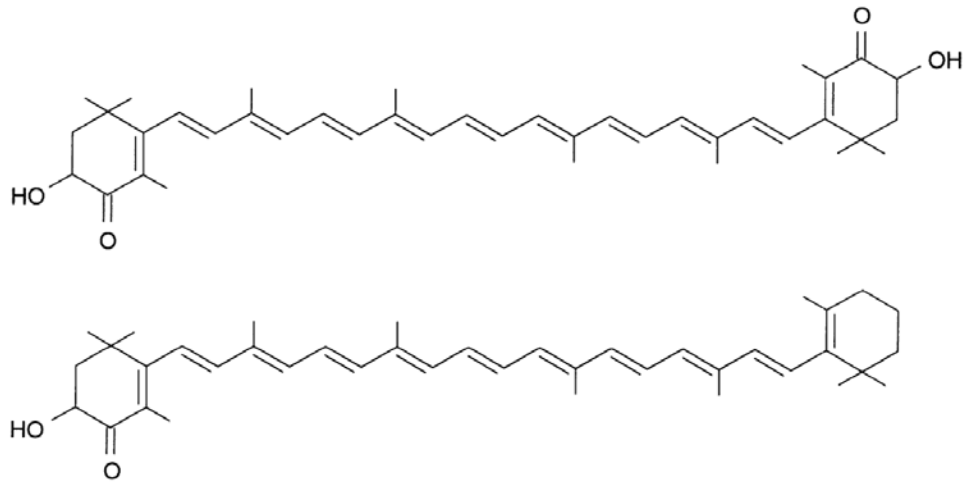

Figure 1.1 Chemical structure of astaxanthin (top) and canthaxanthin (bottom).
Taken from (Shahidi et al., 1998)

supplements sporadically led to ocular lesions in humans, but the consumption of similar amounts can realistically not be reached via the consumption of pigmented salmonid products (Brizio et al., 2013). The potential health benefits from carotenoid consumption on the other hand are considerable. Numerous *in vitro* studies showed highly potent antioxidant activities of Ax and Cx due to modulation of peroxidation, free radical scavenging (Cantrell et al., 2003; Di Mascio et al., 1990; Edwards et al., 2016; Lorenz and Cysewski, 2000; Shimidzu et al., 1996), and protection from DNA damage due to UV radiation (Brizio et al., 2013), enhanced immuno-modulatory functions and augmented cellular gap-junction communication (Edwards et al., 2016; Esatbeyoglu and Rimbach, 2016; Lorenz and Cysewski, 2000) were reported. Health benefits for humans through consumption of animal products containing carotenoids have been suggested (Baker and Günther, 2004) even though the concentrations in salmonid products may be too low to imbue significant human health benefits (Brizio et al., 2013).

In addition, pigments improve organoleptic properties of salmonid flesh (Diler and Gokoglu, 2004). This phenomenon may be rationalised by carotenoids serving as a precursor of certain flavour types or by the modulating chemical reactions that imbue a “salmon aroma” (Yanar et al., 2007).

1.3 Utilisation and retention of dietary carotenoids

The utilisation of dietary carotenoids for flesh deposition in salmon lies between 5.5 (Page and Davies, 2006) and 15-20 % (Rørvik et al., 2010) and is therefore very low in comparison to other nutrients. Efficient deposition of Ax at moderate temperatures (3.5 - 16°C) is best achieved by application of diets with moderate Ax concentration (60 mg/kg) in pelleted feeds during the entire marine phase of production, rather than by application of diets with high concentrations over a short period of time (Torrissen et al., 1995). An increase of the dietary Ax concentration over 60 mg/kg (up to 200 mg/kg) only led to a minor increase in flesh deposition, and vastly reduced the utilisation efficiency of dietary Ax for flesh deposition (Torrissen et al., 1995).

1.4 Factors affecting the utilisation and retention of dietary carotenoids

1.4.1 Absorption and digestion

Dietary carotenoids are available for flesh deposition after entering the blood circulation (Storebakken and Goswami, 1996; Ytrestøyl et al., 2006) from the gastrointestinal tract. It is established that absorption and passage through the gut is a major obstacle for dietary carotenoids to enter the circulatory system, and is also considered a major factor attributing to low utilisation of dietary carotenoids for flesh deposition (Kiessling et al., 2003; Rørvik et al., 2010; Ytrestøyl and Bjerkeng, 2007a; Ytrestøyl et al., 2005; Ytrestøyl et al., 2006). Dietary carotenoids are absorbed from the gut by passive diffusion following liberation from the feed matrix, dispersion in a lipid emulsion, solubilisation into micelles and then moving through the unstirred water layer to the intestine wall (Olsen et al., 2005; Ytrestøyl et al., 2006). They are absorbed in the pyloric caeca and the intestine and the inefficiency of this process may be further exacerbated by oxidative reduction, since carotenoids are highly prone to oxidation once liberated from the feed matrix (White et al., 2003; Ytrestøyl et al., 2006). The absorption of Ax may be affected by *in vivo* isomerisation while travelling through the gastrointestinal tract , as indicated by higher contents of 9Z-isomers in faeces compared to the dietary composition (Ytrestøyl et al., 2005; Ytrestøyl et al., 2006). However, selective absorption of all-E and 13Z-isomers along the gut cavity may be an alternative explanation for the selective accumulation of the 9Z-form in faeces (Ytrestøyl and Bjerkeng, 2007a).

Several dietary factors affect carotenoid absorption-efficiency. Increasing dietary total lipid concentration, supplemented bile salts and cholesterol can improve carotenoid absorption in salmonids possibly due to the pigment's lipohilic nature (Bjerkeng et al., 1999; Chimsung et al., 2014; Nickell and Bromage, 1998; Olsen et al., 2005). Increasing dietary fibre can lead to reduced carotenoid absorption in humans, and mutual inhibition in absorption occurs between different carotenoids in mammals, which is thought to be caused by the competition for solubilisation into micelles (Riedl et al., 1999; Van den Berg, 1999). However, dietary supplementation of 40 mg/kg luthein or zeaxanthin did not reduce the plasma concentrations of Ax (Chimsung et al., 2014). Varying the dietary concentrations (and ratios) of Ax and Cx had no effect upon the subsequent deposition efficiency of the other carotenoid in flesh (Baker et al., 2002; Buttle et al., 2001), suggesting mutual inhibition did not occur.

The apparent digestibility coefficient (ADCT) of pigment carotenoids in salmon is usually between 40 and 50% (Rørvik et al., 2010). One important factor that affects the ADCT of carotenoids in salmon is the level feed intake. This could be due to changes in the passage time of chymus through the GIT with changing feed intake, and an increase in feed intake gives slowly digested nutrients like carotenoids lesser time to be liberated from the feed matrix and to be absorbed (Ytrestøyl et al., 2006). The evacuation rate of chymus was significantly increased in salmon held at 18°C, compared to salmon held at 14°C (Handeland et al., 2008) and the restriction of feeding rates from 1.2% to 0.6% (feed/body weight/day) led to a three-fold increase (11.8% to 32.1%) in the ADCT of carotenoids in large salmon (Rørvik et al., 2010). The negative correlation between feed intake rates and ADCT of Ax is much stronger compared to other nutrients in salmon, making restricted feeding a potential tool to improve pigmentation (Ytrestøyl et al., 2006), which however can cause significantly increased production costs (Einen et al., 1999; Ytrestøyl et al., 2006). Moreover, ADCT of Ax was 10% lower in salmon held at 8°C compared to salmon held at 12°C and differences in the conversion of Ax to different isomers could explain this phenomenon (Ytrestøyl et al., 2005). While travelling through the gut wall, a considerable proportion of Ax and Cx were converted into colourless vitamin A_1 and A_2 precursors in vitamin A-depleted rainbow trout (Schiedt et al., 1985) and in everted trout intestines exposed to an Ax-containing solution (White et al., 2003). Pro-vitamin A activity of Ax was also suggested in Atlantic salmon (Christiansen et al., 1995b). Atlantic salmon administered with a single oral dose of radiolabelled Ax showed high radioactivity in the intestine even ten days after the last meal (Aas et al., 1999). The long presence in the intestinal wall could make Ax more vulnerable for metabolic conversion occurring within the gut wall before Ax reaches blood circulation. The conversion of carotenoids in the gut wall, as well as oxidation and potential metabolic conversion in the gut cavity indicates that the ADCT of carotenoids is a poor indicator of bioavailability. This is in contrast to the concentration blood, which showed good correlation to carotenoid concentration in muscle (Kiessling et al., 2006; Ytrestøyl et al., 2005; Ytrestøyl et al., 2006).

1.4.2 Blood transport, metabolism and deposition, effect of dietary factors and *differences between astaxanthin and canthaxanthin*

After passage through the gut, carotenoids are taken up by lipoproteins and other transport protein in blood (Aas et al., 1999; Lubzens et al., 2003). Differences in hydrophobicity

between carotenoids possibly affect their absorption efficiency into lipoproteins in blood after absorption (Choubert, 2010). However, an *in vitro* incubation of lipoproteins led to a 100 fold increase of Cx in rainbow trout plasma (Chavez et al., 1998). It therefore appears unlikely that inefficient binding of carotenoids to transport-media in blood may limit the availability of dietary carotenoids for flesh deposition. The largest proportion of circulating Ax was found in the lipoprotein deficient plasma (Aas et al., 1999; Chimsung et al., 2013) and albumin may be the main transport protein in salmon plasma (Aas et al., 1999). Lipoprotein classes carrying Ax included the high density lipoprotein, the low density lipoprotein, and the very low density lipoprotein (VLDL) classes (Aas et al., 1999; Chimsung et al., 2013). The concentration of circulating Ax was increased by dietary supplementation of 2% cholesterol, which was ascribed to an increase of VLDL lipoproteins in combination with higher Ax concentrations associated to this lipoprotein class (Chimsung et al., 2013). Idoxanthin (Ix) is the first metabolic product of Ax in salmonids (Schiedt et al., 1985) and Ix appeared six hours after Ax ingestion, indicating that metabolic conversion takes place in blood quickly after absorption (Aas et al., 1999).

The metabolic conversion to colourless products in general is considered a factor that significantly contributes to the low utilisation of dietary pigment carotenoids for flesh deposition and 60-80% of pigment carotenoids absorbed by salmonids may be metabolically transformed and excreted (Ytrestøyl and Bjerkeng, 2007b). Over 90% of all pigment carotenoids in the whole body of Atlantic salmon are deposited in white muscle tissue, and high proportion of the whole body carotenoid pool in the liver indicated that this organ also has carotenoid storage function (Page and Davies, 2006). However, high concentrations of Ix indicate that the liver also plays a significant role in carotenoid catabolism (Bjerkeng and Berge, 2000; Page and Davies, 2003, 2006). The substance 4'hydroxyechinenone is likely the first metabolic product of Cx (Schiedt et al., 1988; Ytrestøyl et al., 2004) in salmon and the metabolic conversion of Ax and Cx is high in small fish and decreases as fish grow (Aas et al., 1999; Ytrestøyl et al., 2004). This relationship could explain why carotenoid concentrations in flesh increase with fish size, which is well established for many salmonid species (Mørkøre and Rørvik, 2001; Olsen and Mortensen, 1997; Torrissen et al., 1989). Following the application of radiolabelled Ax to salmon, the kidney showed radioactivity which could not be ascribed to Ax or Ix, indicating that Ax metabolites may be excreted via the urine (Aas et al., 1999). It is not understood how mechanisms in regard to metabolic transformation and excretion of carotenoids work, or how carotenoid metabolism is

controlled (Ytrestøyl et al., 2005). However, stress may increase the conversion of Ax to Ix (Schiedt et al., 1989).

Carotenoid deposition efficiency in salmon differed between diets with different fatty acid (FA) composition (Bjerkeng et al., 1999; Regost et al., 2004; Rørå et al., 2005a; Rørå et al., 2005b). However, the mechanisms that led to this phenomenon were not elucidated. The deposition of Cx is more efficient than that of Ax in Atlantic salmon (Buttle et al., 2001; Page and Davies, 2006). This may be ascribed to higher rates in hepatic catabolism of Ax compared to Cx, as indicated by higher proportions of the total body Ax pool in liver compared to Cx (Page and Davies, 2006). However, differences in uptake mechanisms of muscle cells between these two carotenoids may also be relevant (Bjerkeng et al., 2007; Page and Davies, 2006), even though these are not well understood.

Differences in cellular uptake mechanisms could also explain why salmonids are able to deposit large amounts of carotenoids into white muscle tissue, in contrast to white-fleshed fish species like Atlantic cod (*Gadus morhua*) (Ytrestøyl and Bjerkeng, 2007a). Both, cod and salmon showed linear a correlation between intraperitoneally administered Ax dose and blood Ax concentration, but the concentration of Ax in the muscle considerably increased only in salmon (Ytrestøyl and Bjerkeng, 2007a). The salmon thereby showed Ax concentrations of up to 30 mg/kg in flesh, which was vastly higher compared to concentrations in studies with salmon following oral carotenoid administration (Ytrestøyl and Bjerkeng, 2007a). Further, the dose-response relationship between intraperitoneally injected Ax dose and flesh concentration was linear, which indicated unsaturated binding capacity of muscle (Ytrestøyl and Bjerkeng, 2007a). This may also indicate that restricted uptake of Ax into muscle cells alone cannot explain the increased efficiency in flesh deposition of dietary Cx compared to Ax in salmon.

1.4.3 Natural vs synthetic carotenoid sources, differences between salmonid species

Aside from synthetic Ax and Cx, a range of natural pigment carotenoid-sources for the supplementation into diets for aquaculture species exist, including herbal products, microalgae, yeasts and crustacean by-products with Ax as the main pigment type (Higuera-Ciapara et al., 2006). Most studies that examined the deposition of pigment carotenoids in salmonids tested synthetic Ax and Cx sources, which probably related to higher industry relevance at the time. However, there has been an increasing demand for aquaculture

products with natural pigment sources (Teimouri et al., 2013) and a number of pigment sources were therefore tested in salmonids in recent years.

Atlantic salmon fed diets with similar Ax-concentrations, using either Ax from *Phaffia rhodozyma* or a synthetic Ax source, showed equal (Whyte and Sherry, 2001) or even higher (Bjerkeng et al., 2007) concentration of Ax in flesh of salmon fed the natural source. Differences in the manufacturing process of the *Phaffia rhodozyma*-source can significantly influence the bioavailability of Ax and may therefore impact on the utilisation efficiency of Ax for muscle deposition (Bjerkeng et al., 2007). The pigmentation efficiencies of natural pigments derived from meal of: red pepper, red chilli (*Capsicum annum*), algae (*Spirulina platensis*), marigold flower, algae and shrimp waste were compared with synthetic Ax in rainbow trout (*Oncorhynchus mykiss*) (Choubert et al., 2006; Diler and Gokoglu, 2004; Ingle de la Mora et al., 2006; Teimouri et al., 2013; Yanar et al., 2007). The results were inconsistent since some studies indicated that synthetic Ax deposition in rainbow trout flesh was deposited more efficiently compared to the natural carotenoids (Diler and Gokoglu, 2004; Ingle de la Mora et al., 2006; Teimouri et al., 2013; Yanar et al., 2007), whereas other studies indicated the opposite (Choubert et al., 2006; Teimouri et al., 2013). In some cases (Diler and Gokoglu, 2004; Ingle de la Mora et al., 2006; Teimouri et al., 2013) the actual pigment carotenoid types and their concentrations in the natural sources were not identified, which did not allow a direct comparison of the deposition efficiency between the natural and synthetic Ax sources. Carotenoids other than Ax and Cx from plant sources that may potentially contribute to salmonid pigmentation are capsanthin and capsorubin (Torrissen et al., 1989). In general, negative side effects of natural pigment sources can be increased yellowness of flesh and heterogeneous pigmentation along the fillet (Teimouri et al., 2013; Yanar et al., 2007), but the plant components that caused the increased yellowness were not identified (Yanar et al., 2007).

Significant differences in the metabolism and utilisation of Ax and Cx between Atlantic salmon and other salmonid species were reported (March and MacMillan, 1996; Page and Davies, 2006). The reasons for these differences are not known, but they may be due to evolutionary origin (Kiessling et al., 2003). Nevertheless, one salmonid species may be a poor model organism for other salmonid species regarding the metabolism and utilisation of carotenoids.

1.4.4 Inter-individual variability and genetic factors affecting pigmentation quality

The inter-individual variability in pigment concentration in flesh of salmon is high, even in salmon of similar weights, exposed to same treatment and fed the same diets (Kiessling et al., 2006) and reasons for this phenomenon appear not well understood. In Tasmania, this phenomenon may even be exacerbated in the summer period (unpublished data). Pigmentation quality is a trait with moderate to high heritability, indicating that selective breeding may be a useful tool to improve pigmentation quality (Bjerkeng, 2000; Powell et al., 2008; Quinton et al., 2005). Fillet colour and carotenoid deposition are genetically correlated to fish size, total lipid concentration and several biometric traits (Bjerkeng, 2000; Powell et al., 2008; Quinton et al., 2005).

1.5 The effects of high temperature on performance and physiology in salmon, effect of fish size

Salmonids are ectotherms and temperature is a major limiting factor for growth, feed intake and survival in teleost fishes (Brett and Groves, 1979; Jørgensen et al., 2014; Underwood et al., 2012). Many teleost fish species are tolerant to temperatures which are considered as very low, whereas an increase of temperature above the preferred range can quickly reach the upper limits with negative consequences for feed intake, growth and short term survival (Brett and Groves, 1979; Katersky and Carter, 2007; Kullgren et al., 2013). Feed intake in teleosts usually increases with increasing temperature, but drops quickly when temperatures approach the upper thermal limit (Jobling, 1994; Katersky and Carter, 2007). The temperature range of 13-15°C is considered as the optimal growth temperature for Norwegian strains of Atlantic salmon in sea water (Jørgensen et al., 2014). Salmon post-smolt held at 18-19°C showed deteriorated growth performance, utilisation of nutrients and increased depletion of energy storage compared to groups held at lower temperature, with increasing severity over time (Handeland et al., 2008; Kullgren et al., 2013). This was ascribed to a shift in lipid metabolism and endocrine alterations at ET including an increase in the concentration of growth hormone and leptin in circulation, where the latter is thought to supress appetite in salmon (Kullgren et al., 2013). Larger salmon (2 kg) held at 19°C showed reduced feed intake, feed utilisation and the depletion of energy storage in concurrence with a decrease in the concentration of plasma ghrelin when compared to a 14°C control treatment (Hevrøy et al., 2012). The reduction of plasma ghrelin may represent a possible mechanism that facilitates a higher propensity to cease feeding intake in salmon held at ET, which may

represent an ET coping strategy (Hevrøy et al., 2012). Anecdotally, a decrease in feed intake is initially observed when the temperature exceeds the preferred ranges of salmon in Tasmania, followed by the cessation of feed intake and ultimately increasing mortality as the temperature continues to rise (Fox-Smith, pers. communication). Ectothermic species generally respond to increasing temperature with increasing metabolic activity and thus, oxygen consumption (Jobling, 1994; Katersky and Carter, 2007; Lushchak, 2011). Aerobic performance can be characterised by the term "aerobic scope", which is calculated as the difference between the standard metabolic rate and the maximal aerobic metabolic rate (Jensen et al., 2017). One possible theory behind the reduced feed intake at ET is reduced aerobic scope (Jobling, 1994; Katersky and Carter, 2007), which may also limit the capacity to metabolise nutrients and may ultimately reduce appetite. Death occurs if temperatures rise further and a critical thermal limit is exceeded (Clark et al., 2011) and death may be caused by cardiovascular collapse (Farrell, 2002). It therefore seems conceivable that starvation at temperature extremes could alternatively be a mechanism that protects salmon from cardiovascular collapse.

In Tasmania, decreasing rates of feed intake and increasing mortality rates as temperature rises appear to affect larger salmon earlier under commercial conditions (Carter et al., 2008). Experimentally, large wild cutthroat trout showed lower tolerance towards ET compared to smaller specimens (Underwood et al., 2012) and larger Barramundi (*Lates calcarifer*) were more susceptible to problems associated with ET and limited levels of dissolved oxygen (Glencross and Felsing, 2006). Furthermore, the comparison of studies that reported the cardio somatic indices (CSI) in Atlantic salmon indicated that CSI may be negatively correlated to fish size (0.18 in small salmon parr (Sigholt et al., 1989), 0.16 in 90 g salmon (Nordgarden et al., 2002), 0.14 in 100 g salmon (Sanden et al., 2006) and 0.13 in 1 kg salmon (Sagstad et al., 2008)). This may be a possible explanation for the negative relationship of fish size and heat tolerance/aerobic scope in salmon.

1.6 Adaptability of salmon to elevated temperature

Populations of sockeye salmon (*Oncorhynchus nerka*) with more challenging migration conditions such as higher temperature showed optimised cardiovascular adaptions like increased heart size (Eliason et al., 2011). Further, different stocks of wild sockeye and Coho salmon (*Oncorhynchus kisutch*) showed different temperature optima which reflected the ambient temperature of the natal streams of a given stock (Lee et al., 2003). Physiological

adaption to higher environmental temperatures in salmonids does thereby not exclusively occur via heredity and evolutionary adaption, but also in individuals after exposure to high temperature even after periods as short as a few weeks (Jørgensen et al., 2014; Underwood et al., 2012). The sea water temperature ranges around Tasmania in the summer period belong to the highest in all global areas with sea water salmon production. The Tasmanian stock of Atlantic salmon originated from the same wild population has been genetically isolated since 1965 (Ward et al., 1994) and is now likely adapted to higher temperatures compared to other stocks (Barnes et al., 2011).

1.7 Ambient temperature and feed intake, implications for pigmentation quality

Tasmanian salmon with sufficient oxygen saturation (> 80%) still perform well at 19°C (Carter et al., 2008). However, I am not aware of a study that tested the pigmentation quality of Atlantic salmon at these temperature ranges. The previous section (1.5) described the pronounced impact of suboptimal temperature on feed intake when temperature exceeds the preferred range. Reduced feed intake increases the ADCT of carotenoids and may be a tool for improved pigmentation (Section 1.4.1). This may be a realistic scenario when feed intake rates of all individual salmon of a population reduce equally. However, little is known about the impact of ET on the feed intake of individual fish. A higher number of salmon held under experimental conditions at 19°C showed reduced feed intake and a much higher number of individuals showed an empty gastrointestinal tract compared to a group held at 14°C (Hevrøy et al., 2012). This may indicate that decreased feed intake at ET could be due to individual fish that cease feeding, rather than consistently reduced feed among the entire population. Asymmetry in feed intake between individuals is also associated with the summer period in Tasmania, indicated by either very full or entirely empty gastrointestinal tract in individual fish sampled during routine commercial control (unpublished data). This may indicate that starvation may be involved in the deteriorated pigmentation in the summer period and could be an explanation for the increased inter-individual differences in pigmentation quality (section 1.4.4). However, pigment depletion in immature salmonids may not be caused by starvation *per se*. Carotenoid concentrations in flesh of rainbow trout were stable after starvation periods of more than twelve weeks (Choubert, 1985; Foss et al., 1984). The Ax concentrations in Atlantic salmon muscle were marginally affected when starved in sea water for 110 days, or compared to fillets of feeding salmon, respectively (Einen et al., 1999; Einen and Thomassen, 1998). Nevertheless, this was the case in fish held at cold or moderate

temperatures (Choubert, 1985; Einen et al., 1999; Einen et al., 1998; Foss et al., 1984), but increasing temperature, and subsequently the elevated level of the basal metabolic rate, due to increased energy demand, may exacerbate carotenoid depletion (Foss et al., 1984). A very high demand for endogenous energy and tissue exists in anadromous salmonids during the life stage of maturation spawning migration (Seiliez et al., 2012). This is due to voluntary starvation in concurrence with the synthesis of gonads and the physically challenging spawning migration. Pigment depletion from muscle was observed in anadromous salmonids during these life stages (Choubert and Blanc, 1993; Doucett et al., 1999; Yamashita and Konagaya, 1990). This may reinforce the hypothesis that increasing temperature may increase the amount of muscle tissue fuelled, which may ultimately exacerbate pigment depletion. Salmon utilise very high amounts of total lipid at starvation and high amounts of total lipid are thereby mobilised from white muscle tissue (Einen et al., 1998). Due to the lipophilic nature of carotenoids and interactions between lipids and carotenoids in salmon (sections 1.4.1, 1.4.2), it appears that flesh deposited lipids could be of particular importance if the mobilisation of nutrients from muscle at starvation facilitates pigment depletion.

1.8 Carotenoids and oxidative stress, interactions with other antioxidants

The term oxidative stress (OS) describes the state of an organism when the level of so called reactive oxygen species (ROS) produced exceeds the level to which ROS are eliminated by the organism. ROS (e.g. superoxide anion, hydrogen peroxide, hydroxyl radical) are thereby inevitably produced by aerobic metabolic activity (Lushchak, 2011). Whereas many antioxidants like glutathione and a range of antioxidant enzymes are produced endogenously. Others like the vitamins A, E and C (or at least their precursors) and carotenoids cannot be synthesised and must therefore be provided from exogenous sources like diet (Lushchak, 2011). There is a range of potential mechanisms as to how the pigment carotenoids can serve as antioxidants (section 1.2), and interactions of carotenoids and other antioxidants exist in salmon. In addition to metabolic conversion from Ax to vitamin A (section 1.4.1), the increase of dietary Ax concentration led to increased concentration of vitamin A in salmon alevins, which showed that Ax has vitamin A protecting properties (Christiansen et al., 1994). Vitamin A itself is an antioxidant vitamin (Christiansen et al., 1995a), which may indicate that interactions between Ax and vitamin A may be relevant under conditions favourable for OS. Atlantic salmon post-smolt held at 8°C showed minor responses in OS indices when diets with different contents of anti- and pro-oxidants, including Ax were fed (Lygren et al.,

1999). None of the other dietary supplements affected the concentration of Ax, which showed that salmon can tolerate a large range of dietary pro- and antioxidants under normal rearing conditions (Lygren et al., 1999). However, this may not be the case when rearing conditions favourable for OS are combined with an impaired antioxidant status (Lygren et al., 1999). In juvenile salmon, the liver concentration of α-tocopherol (A-Toc) was not affected by the concentration of ascorbic acid, until deficiency of ascorbic acid was reached, which led to a marked reduction of hepatic A-Toc concentration (Hamre et al., 1997). This showed that ascorbic acid can regenerate A-Toc and this required a minimum concentration of ascorbic acid (Hamre et al., 1997). Similar interactions were also observed between Ax and A-Toc in salmon (see chapter 2). Hence, a balanced antioxidant status in salmon highly important and the depletion of one antioxidant can quickly lead to the depletion of other antioxidants.

1.9 Interconnections between oxidative stress, starvation and temperature

Feed restriction can lead to the depletion of antioxidants in mammals (Godin and Wohaieb, 1988). This scenario appears conceivable to be involved in the reduced pigmentation quality in salmon during summer, when the supply of exogenous antioxidants like carotenoids ceases due to starvation and the requirement for antioxidants increases with increasing OS. Increasing temperature leads to an increase of OS in ectotherms, where increased metabolism leads to increased production of ROS (Lushchak, 2011). Oxygen consumption in fish is a measure of metabolic activity and the oxygen consumption increases with the amounts of feed consumed (Forsberg, 1997). Paradoxically, numerous studies with teleost fish demonstrated an increase of OS at starvation (Chapter 5).

1.10 Aim, hypotheses and objectives

To date, numerous studies have examined pigmentation in salmonids or physiological aspects of salmonids at ET. However, despite the broad knowledge now available around both of these areas, there is still a lack of fundamental knowledge about pigmentation, and the metabolic fate of pigment carotenoids in salmon exposed to ET. The sea temperatures in global areas with salmon farming increased in recent years (Hevrøy et al., 2012) and climate researchers predict a further rise in global sea temperatures in the future. Hence, more knowledge about the consequences of ET on the product quality of salmon is required (Battaglene et al., 2008), including knowledge about the causes of reduced pigmentation quality.

The overall aim of the thesis was therefore to improve the understanding of factors and metabolic processes that may contribute to reduced pigmentation quality in salmon exposed to elevated temperature. This is to improve the pigmentation management in salmon farms exposed to elevated temperature.

The previous sections outlined factors that could be relevant for summer pigmentation. Naturally, the effect of ET is at the centre of the current research. However, ET has a pronounced attenuating impact of ET on feed intake and can even lead to starvation (Sections 1.1, 1.5, 1.7). Under certain circumstances, starvation itself can be detrimental for pigmentation in salmonids (Section 1.7). The dynamics of the issue could change dramatically depending on whether deteriorated pigmentation is due to the deteriorated pigment retention when fish are still feeding, or the depletion of carotenoids at starvation.

Since the effects of sub-optimum temperatures on pigment deposition in Atlantic salmon have not yet been investigated (Section 1.7), the deteriorated pigmentation quality could be simply due to the deteriorated retention of carotenoids in muscle. Possible mechanisms that may lead to this could be an increased level of OS due to increased ROS production due to elevated metabolic activity (Section 1.9). However, it seems conceivable that elevated metabolic activity may also lead to an upregulation of the metabolic conversion of pigment carotenoids into colourless products (Section 1.4.2). Due to interactions of dietary carotenoids and lipids (Section 1.4.2), it appears that changes in the interactions between dietary lipids and carotenoids during absorption, transport or deposition at ET could be involved.

Starvation itself did not cause pigment depletion in starving salmonids (Section 1.7), but increasing temperature increases the metabolic activity and it was hypothesised that increasing starvation metabolism may affect pigment depletion (Section 1.7). Hypothetically, the decrease of metabolic activity at starvation (Section 1.9) may alleviate the metabolic conversion from carotenoids into colourless products also. However, despite reduced metabolic activity, the level of OS is usually increased in starving teleosts (Section 1.9) which may exacerbate pigment depletion due to their use as antioxidants. Furthermore, the muscle tissue not only accommodates the majority of carotenoids deposited in salmon (Sections 1.4.2), but is also the main tissue that salmon use as energy and nutrient storage. It may therefore be possible that the mobilisation of nutrients could lead to carotenoid depletion

at starvation. Due to the lipophilicity of carotenoids and interactions with dietary lipids (Sections 1.4.1, 1.4.2), the mobilisation of lipids may be of importance in this context.

The main hypotheses are therefore:

- Elevated temperature causes impaired pigmentation quality in Atlantic salmon.

- This may be due to both; decreased pigment carotenoid deposition in feeding, and pigment depletion in starving salmon, respectively

- In both scenarios, impaired pigmentation quality may be due to: the reduction of pigment carotenoids due to their use as antioxidants, increased metabolic conversion of pigment carotenoids into colourless products, or altered interactions with lipids at elevated temperature

The objectives of the study were to:

- Examine the effects and interactions of the factors elevated temperature and starvation on pigmentation quality

- Test the relationship of oxidative stress and pigmentation quality at elevated temperature

- Elucidate the effects of lipid deposition into muscle in feeding, and lipid mobilisation from muscle in starving salmon at elevated temperature

- Examine the effect of elevated temperature on the metabolic conversion of carotenoids into colourless products

- Explore dietary strategies involving the manipulation of dietary antioxidants and lipids that may alleviate reduced pigmentation quality at elevated temperature

1.11 Use of experimental fish for different chapters and style of the thesis

Two experiments were conducted for the following research-chapters (2-5). Both experiments consisted of a growth (or nutrient-loading) and a starvation period. Chapter 2 addressed both periods of the first experiment. Chapter 3 addressed the growth-period of the second experiment and the chapters 4 and 5 addressed different aspects of the starvation period of the second experiment. Each of the research chapters was written as the basis for a manuscript. This approach led to some repetition, especially in the materials and methods and introduction sections.

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CHAPTER 2

Pigment depletion at an elevated temperature and starvation in Atlantic salmon (*Salmo salar*) post-smolt is not influenced by dietary carotenoid type and α-tocopherol concentration

2.1 Abstract

Fillet pigment depletion in Atlantic salmon arises after periods of elevated water temperatures and associated to the cessation of feed intake. The study tested whether dietary pre-loading with different pigment carotenoids (astaxanthin; Ax and/or canthaxanthin; Cx) combined with two dietary α-tocopherol (A-Toc) concentrations (500 and 1000 mg/kg) could inhibit pigment depletion in Atlantic salmon when challenged with starvation at elevated temperature *in vivo*. We also tested if oxidative stress (OS) manifests as an underlying depletion mechanism. Carotenoid concentrations in whole fillet homogenates were not significantly decreased post-challenge and fillet A-Toc concentration was significantly increased in contrast to significantly decreased OS indices. However, image analysis revealed localised fillet pigment depletion that was not associated to carotenoid type or A-Toc concentration. These data imply that localised pigment depletion to fillets at elevated temperature and starvation was not prevented by pre-loading varying carotenoid and A-Toc concentrations. Further, we suggest that OS may not facilitate pigment depletion *in vivo*.

2.2 Introduction

Atlantic salmon production is one of the fastest growing food industries worldwide and has been introduced to countries far from this species' natural range. In recent years, global warming has led to elevated sea water temperatures, affecting global sites of salmon production (Hevrøy et al., 2012). Sea water temperatures at some Tasmanian sites of production commonly exceed 19°C for prolonged periods (Battaglene et al., 2008) and are above the ideal temperature range for Atlantic salmon production in sea water (Hevrøy et al., 2012; Kullgren et al., 2013; Miller et al., 2006).

Pigmentation of farmed salmon is enabled by deposition of carotenoid pigments such as astaxanthin (Ax) and canthaxanthin (Cx) into flesh (Rørvik et al., 2010). Choice experiments have shown that consumer willingness to pay for salmon products is strongly linked to the red colour intensity (Alfnes et al., 2006) which shows good correlation to carotenoid pigment concentrations in flesh (Buttle et al., 2001). Further, Ax and Cx are highly beneficial for human health particularly due to antioxidant properties (Guerin et al., 2003; Palozza and Krinsky, 1992). *In situ* observations of harvest-sized salmon that have experienced temperature extremes in Tasmania include the cessation of feed intake and reduced pigmentation quality, characterised by generally reduced red colour intensity and

increasing pigmentation heterogeneity on the fillet surface (T. Fox-Smith, pers. communication).

As temperature rises, metabolic processes in fish are elevated, which can lead to oxidative stress (OS) (Lushchak, 2011) and periods of elevated OS have been associated with decreased concentration of Ax in salmon flesh (Nordgarden et al., 2003). It was therefore hypothesised that reduced pigmentation quality at temperature extremes is due to the metabolic use of carotenoids as antioxidants. Previous studies revealed considerable OS related interactions between Ax and the antioxidant α-tocopherol (A-Toc) in salmon. This was indicated by interactive sparing (Bell et al., 2000; Christiansen et al., 1995) and suppression of Ax catabolism with increasing dietary concentration of A-Toc (Bjerkeng et al., 1999). Among other tocopherols and tocotrienols, A-toc is associated with the generic term vitamin E, of which the highest vitamin E activity is exhibited by A-Toc in animals (Hamre, 2011). This may indicate that high concentrations of A-Toc in muscle could be beneficial in the prevention of OS related Ax depletion. Very little information on oxidative stress related interactions between A-Toc and Cx in salmon is available.

The concentration of A-Toc can be readily manipulated in several salmon issues via manipulation of dietary A-Toc concentrations (Bjerkeng et al., 1999; Faizan et al., 2013). One strategy in the prevention of carotenoid depletion might therefore be the loading of salmon muscle with high concentrations of A-Toc before periods of temperature extreme lead to cessation of feed intake and consequently the cessation of dietary A-Toc supply.

Global sea temperatures are anticipated to further increase and there is a general lack of knowledge on the effect of elevated temperature on farmed Atlantic salmon, including effects on flesh quality (Battaglene et al., 2008). The present study therefore tested the effects of elevated temperature (19.5°C) and starvation (challenge-phase) on pigment depletion in flesh and indices of OS in salmon. In order to test if Ax and Cx are thereby depleted differently and if high A-Toc concentrations in muscle may prevent carotenoid depletion, different groups of salmon were fed diets with three carotenoid inclusion types at two concentrations of dietary A-Toc under ideal growing conditions (loading-phase) before the challenge-phase commenced.

2.3 Materials and methods

2.3.1 Experimental diets

Experimental diets were formulated to contain the three carotenoid inclusion types: 100 mg/kg Ax (Ax), 50 mg/kg Ax with 50 mg/kg Cx (Mix: M) and 100 mg/kg Cx (Cx) and the A-Toc concentrations: 500 mg/kg (500E) and 1000 mg/kg (1000E), leading to six diets: Ax500E, M500E, Cx500E, Ax1000E, M1000E and Cx1000E (Table 2.1). The synthetic carotenoids were incorporated into microcapsules, containing plant starches and antioxidants. The main source of α-tocopherol was supplemented DL-α-tocopheryl acetate. Minor amounts of the vitamin E-homologues ß-tocopherol and γ-tocopherol were also present from the raw

Table 2.1 Ingredients, carotenoid and α-tocopherol concentrations and chemical composition of the experimental diets

	Ax500E	Mx500E	Cx500E	Ax1000E	Mx1000E	Cx1000E
Component (g/kg)						
Soy protein concentrate	300	300	300	300	300	300
Fish meal	150	150	150	150	150	150
Wheat gluten	136.3	136.3	136.3	136.3	136.3	136.3
Faba beans whole	100	100	100	100	100	100
Rapeseed oil	102.2	102.2	102.2	102.2	102.2	102.2
Fish oil	102.2	102.2	102.2	102.2	102.2	102.2
Wheat	81.3	81.3	81.3	80.3	80.3	80.3
Vitamins, minerals, yttrium oxide	26	26	26	26	26	26
DL-α-tocopheryl acetate (50%)[1]	1	1	1	2	2	2
Astaxanthin (10%)[2]	1	0.5	0	1	0.5	0
Canthaxanthin (10%)[3]	0	0.5	1	0	0.5	1
Carotenoid & tocopherol content (mg/kg)						
Astaxanthin	95.8	48.3	2.2	96.6	50	1.4
Canthaxanthin	3.7	49.8	97.2	4.3	47.4	94.1
Astaxanthin + Canthaxanthin	99.5	98.1	99.4	100.9	97.4	95.5
α-tocopherol acetate	524	499	483	929	959	931
γ-tocopherol acetate	32	N.d.[4]	N.d.	N.d.	N.d.	N.d.
ß, δ-tocopherol acetate	<0.1	N.d.	N.d.	N.d.	N.d.	N.d.
Chemical composition (g/kg, as is)						
Crude protein	425	422	416	422	424	421
Lipid	252	259	249	259	259	265
Moisture	63.1	66.7	65.2	67.5	61.3	63.8
Ash	51.8	49.8	50.3	51.1	50.5	52.8

[1]50%-Lutavit® E-50, BASF
[2]Essention pink, NHU
[3]Carophyll red, DSM
[4]Not determined

materials. Diets were extruded (Wenger TX-57, extruding temperature: 75°C), full sinking pellets (4 mm) and imported (Skretting ARC feed technology plant, Stavanger, Norway).

2.3.2 Fish husbandry and experimental design

The experiment was conducted at the University of Tasmania's (UTAS) aquaculture research facility (Launceston, Tasmania). The UTAS Animal Ethics Committee in accordance with the "Australian code for the care and use of animals for the scientific purposes" approved the care and use of fish for this experiment (approval number A0014015). Female Atlantic salmon (180 g, diploid) held in outside ponds under ambient lighting conditions were transferred from a commercial salmon hatchery (Petuna, Cressy, Tasmania) to an experimental recirculating aquaculture system (RAS) (24 x 350L tanks) equipped with solids filtration, biological filtration, foam fractionation, UV disinfection and temperature control. Fish were divided equally among the tanks then habituated and smolted in the RAS over seven weeks in freshwater (pH 7.0 ± 0.2) under constant lighting and incrementally increased temperature (initial 7°C: final 15°C). Fish were fed to satiation with commercial transfer diets (Skretting, Cambridge, TAS, Australia). RAS salinity was increased one-week prior feeding experimental diets to 28‰ ± 1 and reduced to 22‰ ± 1 after 24 days of feeding for the duration of the trial. Other water quality parameters were maintained with safe limits likewise for the trial duration ($NH_4/NH_3 \leq 1$ mg/L, $NO_2 < 2$ mg/L), dissolved oxygen saturation 80-90%, system water exchange 20%/day (tank water exchange ≈ 1.8 volumes/h). Fish (n = 20) were re-allocated to each tank at the start of the experiment (average fish weight in tanks 214 g ± 4.4 SD). Each diet was randomly allocated to four tanks. During the initial loading-phase fish were hand fed to satiation twice daily. Uneaten pellets were collected from each tank and dried and weighed to determine daily feed intake over 80 days. Following completion of the loading-phase, a challenge-phase commenced during which time the fish were starved, water temperature increased incrementally to 19.5°C over five days and then maintained at that temperature for 23 days.

2.3.3 Sample collection

Tissue samples were taken after the loading-phase (pre-challenge) and the challenge-phase (post-challenge). Fish were sedated in their tanks (10 µL/L clove oil), biomass weighed and counted, six selected blindly and anaesthetised until unresponsive with clove oil (40 µL/L) and killed by a blow to the head. Fish were individually weighed and measured, bled in an ice

slurry for at least 10 min. Livers were excised, weighed and immediately frozen in liquid nitrogen and stored at -80°C until analysis for glutathione (GSH) and glutathione disulphide (GSSG). Excised fillets were boned and wiped clean and dry before images were captured (Section 2.5). Subsequently, fillets were skinned, homogenised with a kitchen processor for 11 sec and subsamples were frozen at either -80°C until analysed for Ax, Cx, A-Toc and malondialdehyde (MDA), or stored at -20°C until analysed for chemical composition. Diets were stored at -20°C before analysis for carotenoids, tocopherols as well as chemical composition.

2.3.4 Chemical analysis

All chemical analyses for the 288 individually sampled fish were conducted as single replicates, diet analyses in duplicate.

Carotenoids in white muscle

Pre-homogenised white muscle was thawed before 1 g was transferred into 7 mL Precellys standard tubes containing 2.8 mm ceramic beads (lysing kit CK28, Bertin Technologies, France) and 4 mL of acetone was added. After cooling the tubes for 30 min at -20°C, the mix was homogenised (Precellys tissue homogeniser, model 24) by 3 cycles of 15 seconds at 6,500 rpm and then centrifuged at 3,000g for 5 min. Subsequently, 2 mL of the supernatant was transferred into a fresh vial and samples were concentrated under N_2-gas at 50°C before the sample was dissolved in 2 mL of a n-hexane/acetone mixture (86:14, v/v). Ax and Cx were analysed on the same analytical run via by normal Phase HPLC (model 1200, Agilent) and subsequent UV/VIS-detection at 470 nm wavelength as described previously (Schüep and Schierle, 1995) with a detection limit of ≤ 0.1 mg/kg. The relative retention time of Cx was 0.35 in relation to Ax and the instrument setup was calibrated using all-E Ax and all-E Cx standards (LGC Standards GmgH, Wesel, Germany). The standards were dissolved in chloroform (60 mg/mL), then diluted (1:20) in either n-hexane for Ax, or cyclohexane/dichloromethane (98:2, v/v) for Cx, respectively, yielding in a final standard concentration of about 3 mg/L. The standard concentrations were confirmed by spectrophotometry (Cary 50, Varian) using E1%/1cm values of 2100 for Ax and 2200 for Cx, respectively at 470 nm absorbance.

Glutathione and glutathione disulphide in livers

About 1 g of liver tissue was transferred in a vial containing 4 mL of ice cold 1.15% (w/w) KCl solution, then homogenised for 15 s (IKA Ultra Turrax T10, basic). After homogenisation, the remaining extraction steps were conducted as described previously (Bouligand et al., 2006), using 50 µL of a stable isotope standard solution containing GSH (Glycine-13C2, 98%+; 15N, 96-99%; 65-70% Net; conc.: 60 µg/mL) and GSSG (Glycines-13C2, 98%+; 15N, 96-99%; 65-70% Net; conc.: 130 µg/mL, both standards were purchased from Novachem Pty Ltd, Collingwood, VIC, Australia). Extracts were stored at -80°C until analysed. The thiol GSH (and surrogate standard) were analysed as their carboxymethyl (CM) derivatives, while GSSG (and surrogate standard) were analysed as free compounds. Analyses were conducted using a Waters Acquity H-Class UPLC instrument coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was used, held at 35°C. The mobile phase consisted of 0.1 % (v/v) formic acid (solvent A) and acetonitrile (solvent B). Initial conditions were 100% A, decreasing to 20% A by 3.5 min, which was maintained for 0.5 min before returning to 100% A over 0.5 min and re-equilibration for 3 min. The flow rate was 0.35 mL/min. The injection volume was 10 µL. The MS/MS was operated in positive ion electrospray mode, with capillary voltage of 2.7 KV, and individual cone voltages / collision energies for each multiple reaction monitoring (MRM) transition. The desolvation temperature was 450°C.

Alpha-tocopherol in white muscle

About 150 mg white muscle was transferred into a glass vial on ice, containing 2 mL of 1% ascorbic acid in ethanol (w/w). Then, 20 µL of a deuterated stable isotope surrogate standard solution (±-α-tocopherol-D6; Novachem, Collingwood, VIC, Australia; conc.: 10 µg/mL) was added. This mixture was homogenized for 30 s (IKA Ultra Turrax T8) on ice, before 700 µL H_2O and 300 µL saturated aqueous KOH was added and the remaining extraction steps were conducted as described previously (Faizan et al., 2013). Extracts were frozen at -80°C until analysed. UPLC-MS/MS conditions were identical to Section 2.4.2, apart from the following. The mobile phase consisted of methanol (solvent A), acetonitrile (solvent B) and isopropanol (solvent C). Initial conditions were 50% A, 50% B, held for 1.5 min. Solvent B was then reduced to 44% by 3.0 min, with an increase in solvent C to 6%. Over the next 0.1 min, conditions were changed to 50% A, 35% B and 15% C, and held for 1.9 min before

returning to initial conditions and re-equilibration for 3 min. Sample injection volume was 4 µL.

Malondialdehyde in white muscle

About 250 mg white muscle was transferred into a glass vial on ice containing 2 mL of 1% (v/v) sulphuric acid conc. in PBS, before 20 µL of a deuterated stable isotope surrogate standard (MDA-D2) solution was added (concentration 5.0 µg/mL). The MDA-D2 surrogate standard was derived from hydrolysis of 1,1,3,3-tetraethoxypropane; 1, 3-D2 (purity: 95%; Novachem, Collingwood, VIC, Australia) with 1% (v/v) sulphuric acid at room temperature in the dark for 2 h. This mixture was subsequently homogenised for 30 s (IKA Ultra-Turrax T10 basic), before 1 mL of homogenate-solution was transferred into a snap-cap vial and centrifuged at 10000 rpm for 30 min at 4°C. The remaining extraction steps were conducted as described previously (Faizan et al., 2014), using a 1.2 mM dinitrophenylhydrazine (DNPH) in acetonitrile/formic acid (98/2, v/v) for MDA and surrogate standard derivatisation prepared fresh daily. Extracts were stored at -80°C until analysed. MDA and surrogate standard (MDA-D2) were analysed as their DNPH derivatives. UPLC-MS/MS conditions were identical to section 2.4.2, apart from the following. The mobile phase consisted of 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). Initial conditions were 60% A, held for 1 min before a gradient to 20% A by 3 min, held for 1 min, prior to returning to initial conditions and re-equilibration for 3 min.

Chemical composition of white muscle and diets

Moisture content was determined gravimetrically by freeze drying to constant weight, total lipid was analysed according to Bligh and Dyer (1959) and ash was determined by sample combustion at 600°C for 6 h. The Kjehldahl method was used to measure nitrogen (N), using sulphuric acid (>98 %) and a copper catalyst for digestion (400°C for 2 h). Crude protein (CP) was calculated as = N × 6.25.

Carotenoids in diets

About 2 g of the ground diets was mixed with 100 mg butylated hydroxytoluene in 6 mL of de-ionised H_2O, shaken and subsequently sonicated (150 W at 35 kHz) in a water bath at 50°C for 30 min. Then, 40 mL of ethanol was added, shaken, before 50 mL of

dichloromethane was added and shaken again. The mixture was left in the dark for 2 h, before dichloromethane was added until a total volume of 100 mL was reached and the flask was shaken again. Subsequently, 25 mL of the extract was purified by elution with 120 mL n-hexane/diethyl ether (50/50, v/v) on a chromatography tube (20 mm diameter) equipped with stopcock, glass filter and 5 g of silica gel. After evaporating the solvents under reduced pressure at 50°C the sample was dissolved in 5 mL n-heptane. Injection volume for analysis was 20 µL using normal phase HPLC with subsequent UV/VIS-detection at 470 nm wavelength. For Ax, a LiChrosorb Si 60 column (5 mm × 125 mm × 4 mm) was used and n-heptane/acetone (86/14, v/v) was used as mobile phase at a flow rate of 1.2 mL/min. For Cx, a Hichrom S5CN column (5 mm × 250 mm × 4.6 mm) was used and n-heptane/acetone/methanol (79.9/20/0.1, v/v/v) was used as mobile phase at a flow rate of 1.5 mL/min. The columns were held at room temperature.

Tocopherols in diets

About 20 g of ground feed was mixed with 130 mL of ethanol, 1 g ascorbic acid, 27 mL potassium hydroxide solution (60/40, w/v) and 50 mg sodium sulphide and stirred for 30 min. Then, the mix was heated to 85°C for 30 min under stirring and regular shaking. The mix was then cooled and filtered, before 40 mL of filtrate was transferred into a 50 mL volumetric flask, then filled to volume with ascorbic acid solution (20/80, w/v). After shaking, 15 mL of this mixture was transferred onto a Chem-elut column (EXtrelut® NT, Merck Millipore) for adsorption. Tocopherols were then eluted by adding 50 mL of cyclohexane. Sample injection volume was 20 µL. Normal phase HPLC (pump: Jasco PU-2080 plus) with subsequent fluorescence detection (Jasco FP-920) at 295 nm wavelength and 330 nm emission wavelength was used for analysis. The column used was a Hichrom Alltima (4.6 mm × 250 mm × 5 µm) and 3.2% ethanol in cyclohexane (v/v) as used as mobile phase at a flow rate of 0.8 mL/min.

2.3.5 Image analysis

Digital images of fillets were taken in a light proof box, modified from a setup described previously (Folkestad et al., 2008). The plywood box (570 mm L × 520 mm W × 630 mm H) was equipped with four halogen light bulbs (55W, Osram, Germany) located at the corners of the ceiling and a light diffusion plate between the light bulbs and the bottom plate. An opening on the top of the box secured the camera lens (focal length 18-24 mm, diameter 58

mm, f16) which was focused on the bottom plate and surrounded by a PVC pipe to prevent direct light irradiation from the light bulbs. All openings were sealed to prevent exterior light contamination. The light settings of the camera used (Canon EOS 400D, resolution max: 3888 x 2592 pixels) were fluorescent light and ISO 100. The bottom plate could be removed through an opening on the side of the box to place fillets. The "line tool" of the image analysis software package (Image Pro Premier 9, Media Cybernetics Inc., Rockville, MD, USA) was used to capture the RGB values of a 32 pixel wide straight line that ran from the most dorsal anterior point to the posterior midpoint of the fillet above the lateral line (Figure 2.1). The transect line was divided into six equal sections and the average RGB values of the first (anterior) and the fourth section which partially covered the dorsal part of the Norwegian quality cut were converted into the CIEL*a*b* colour space, assuming a 2° observer angle and daylight illumination (D65).

2.3.6 Calculations and statistical analysis

DM feed conversion ratio (FCR) was calculated as: feed intake (g DM) / weight gain (g). Specific growth rate (SGR) expressed as % body weight gain/day was calculated as: [(Log_n final fish weight (g) – Log_n initial fish weight (g)) / feed days] × 100. DM feed intake expressed as % of BW per day was calculated as: 100 × (feed intake (g DM) / feed days) / [(initial fish weight (g) – final fish weight (g)) / 2]. Condition factor (K) was calculated as: body weight (g) / body length $(\text{cm})^3$ × 100. Hepatosomatic index (HSI) was calculated as: liver weight (g) / body weight (g) × 100. Total glutathione (tGSH) and oxidative stress index (OSI) were calculated as GSH equivalents as described previously (Bagnyukova et al., 2007). Tanks were the unit of statistical assessment and results are reported as treatment means ± standard error mean (SEM). Statistics software used was SPSS version 22.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). Performance parameters in the loading-phase were assessed using two way-ANOVA, fixed factors: dietary carotenoid inclusion type and concentration of A-Toc. Comparisons of parameters between the two sampling times pre- and post-challenge were assessed using three way ANOVA, fixed factors: Sampling time, dietary A-Toc concentration and dietary carotenoid inclusion type. Normality and homogeneity of variance was ensured by examination of residual plots. The level of significance was set at $p \leq 0.05$.

2.4 Results and Discussion

2.4.1 General observations, performance in the loading phase

The difference between the planned total carotenoid concentrations and the analysed carotenoid concentrations was on average 1.8 % and therefore negligible from a biological point of view, due to the relatively high concentrations tested. Overall, fish more than doubled initial weights in the loading phase and dietary carotenoid inclusion type or A-Toc cocentration did not significantly affect final weights, feed intake, SGR and feed conversion (Tables 2.2, 2.3). About 15 % of fish in three tanks affiliated to the treatments M500E, M1000E and Cx1000E showed light skin lesions after 24 days of feeding. Salinity was therefore reduced and maintained at 22‰ from this day in order to prevent any deterioration. In addition, the fish in the three affected tanks were occasionally fresh water bathed under light sedation (duration up to 4 h) between 24 and 66 days of feeding. Lesions of all fish affected were healed by day 66 and performance of the affected tanks was not significantly different when compared to the other tanks of the respective treatment.

2.4.2 Carotenoid concentration in muscle and fillet image analysis

There was no Cx in the muscle of fish fed the Ax-only diets, and the same is the case for Ax in fish fed the Cx-only diets (Table 2.3). This showed that eventual remainders of these carotenoids in fish muscle at the start of the loading phase were negligible and did not affect the final results. Carotenoid deposition in salmon is strongly correlated to fish size and carotenoid concentrations in flesh of harvest fish are usually between 4-10 mg/kg (Torrissen et al., 1995). The common harvest size of Atlantic salmon is 2-4 kg, which shows that the final carotenoid concentrations of 2.21 – 3.45 mg/kg in our study (Table 2.3) were high in

Table 2.2 Feed intake, specific growth rate and feed conversion of post-smolt Atlantic salmon fed diets with varying carotenoid inclusion types and α-tocopherol concentrations for 80 d

	[1]Ax500E	M500E	Cx500E	Ax1000E	M1000E	Cx1000E
Feed intake[2]	0.98 ± 0.008	0.90 ± 0.022	0.98 ± 0.027	0.93 ± 0.042	0.94 ± 0.025	0.94 ± 0.028
SGR	1.26 ± 0.029	1.14 ± 0.060	1.27 ± 0.086	1.21 ± 0.112	1.24 ± 0.045	1.21 ± 0.076
FCR (DM)[3]	0.84 ± 0.012	0.84 ± 0.022	0.88 ± 0.027	0.86 ± 0.041	0.84 ± 0.015	0.84 ± 0.021

[1]Diets were supplemented with either 100 mg/kg astaxanthin (Ax), 100 mg/kg canthaxanthin (Cx) or a mix of 50 mg/kg Ax and 50 mg/kg Cx (M); at 500 and 1000 mg/kg α-tocopherol (500E, 1000E), respectively
[2]Expressed as % of body weight per day
[3]Feed conversion ratio dry matter basis

Table 2.3 Weights (g), hepatosomatic indices (HSI), condition factor (K), as well as concentrations of carotenoids (mg/kg), α-tocopherol (mg/kg), malondialdehyde (nmol/g) and chemical composition (g/kg) in white muscle of Atlantic salmon post-smolt after fish were fed diets with varying carotenoid inclusion types and α-tocopherol concentrations for 80 d (pre-challenge) and a subsequent phase of starvation at elevated temperature for 28 d (post-challenge), respectively.

		Weight[2]	HSI[3]	K	Ax	Cx	Ax + Cx	A-Toc	MDA	Dry matter	Crude protein	Total lipid	Ash
Pre-challenge	[1]Ax500E	575 ± 10.2	1.17 ± 0.001	1.39 ± 0.025	2.21 ± 0.043	B.d.[4]	2.21 ± 0.043	32.2 ± 2.30	15.2 ± 1.24	275 ± 0.3	200 ± 0.2	55.2 ± 0.40	17.3 ± 0.03
	M500E	526 ± 22.3	1.20 ± 0.077	1.35 ± 0.023	1.45 ± 0.030	1.25± 0.037	2.70 ± 0.063	30.5 ± 1.37	15.2 ± 1.77	274 ± 0.1	206 ± 0.2	50.0 ± 0.10	17.5 ± 0.07
	Cx500E	583 ± 40.2	1.21 ± 0.041	1.31 ± 0.004	B.d.	3.45 ± 0.279	3.45 ± 0.279	33.5 ± 1.53	14.7 ± 1.24	275 ± 0.1	201 ± 0.2	51.1 ± 0.24	16.0 ± 0.01
	Ax1000E	560 ± 49.7	1.28 ± 0.061	1.33 ± 0.013	2.21 ± 0.131	B.d.	2.21 ± 0.131	40.3 ± 1.66	15.5 ± 1.37	269 ± 0.2	199 ± 0.3	50.1 ± 0.10	15.4 ± 0.07
	M1000E	564 ± 23.4	1.29 ± 0.074	1.35 ± 0.019	1.29 ± 0.074	1.09 ± 0.060	2.48 ± 0.130	46.9 ± 2.83	15.4 ± 2.00	269 ± 0.1	201 ± 0.2	48.1 ± 0.19	16.0 ± 0.02
	Cx1000E	557 ± 39.3	1.22 ± 0.076	1.36 ± 0.036	B.d.	3.12 ± 0.191	3.12 ± 0.191	43.8 ± 4.18	15.1 ± 1.58	271 ± 0.3	200 ± 0.2	51.4 ± 0.33	16.9 ± 0.04
Post-challenge	Ax500E	492 ± 23.7	0.75 ± 0.045	1.21 ± 0.034	2.15 ± 0.210	B.d.	2.15 ± 0.210	42.4 ± 3.57	13.3 ± 0.52	278 ± 0.2	206 ± 0.1	52.5 ± 0.20	16.6 ± 0.05
	M500E	417 ± 28.7	0.72 ± 0.033	1.21 ± 0.039	1.17 ± 0.059	1.12 ± 0.052	2.29 ± 0.107	37.9 ± 2.07	12.1 ± 1.24	269 ± 0.1	204 ± 0.3	46.6 ± 0.25	16.9 ± 0.03
	Cx500E	505 ± 47.8	0.71 ± 0.026	1.25 ± 0.015	B.d.	3.08 ± 0.289	3.08 ± 0.289	47.9 ± 4.37	14.1 ± 1.44	277 ± 0.5	204 ± 0.2	54.7 ± 0.46	16.0 ± 0.06
	Ax1000E	469 ± 55.5	0.79 ± 0.026	1.21 ± 0.034	1.93 ± 0.142	B.d.	1.93 ± 0.142	60.9 ± 3.27	13.1 ± 0.41	270 ± 0.2	206 ± 0.1	46.1 ± 0.21	16.8 ± 0.08
	M1000E	492 ± 36.4	0.76 ± 0.030	1.17 ± 0.012	1.17 ± 0.133	1.13 ± 0.136	2.31 ± 0.269	62.4 ± 6.25	13.8 ± 0.90	272 ± 0.3	204 ± 0.1	50.9 ± 0.33	16.4 ± 0.05
	Cx1000E	466 ± 35.2	0.76 ± 0.016	1.22 ± 0.016	B.d.	3.45 ± 0.180	3.45 ± 0.180	54.6 ± 3.43	11.9 ± 0.54	270 ± 0.5	207 ± 0.1	45.2 ± 0.59	15.6 ± 0.02
3 Way-ANOVA													
Sampling time		***.	***	***	N.s[5]	N.s	N.s	***	*	N.s.	**	N.s	N.s
α -tocopherol conc.		N.s	N.s	N.s	N.s	N.s	N.s	***	N.s	N.s	N.s	N.s	N.s
Carotenoid inclusion		N.s	N.s	N.s	***	***	***	N.s	N.s	N.s	N.s	N.s	N.s

[1]Diets were supplemented with either 100 mg/kg astaxanthin (Ax), 100 mg/kg canthaxanthin (Cx) or a mix of 50 mg/kg Ax and 50 mg/kg Cx (M); at 500 and 1000 mg/kg α-tocopherol (500E, 1000E), respectively

[2]Initial average tank weights: 214 g ± 0.91 SEM

[3]HSI = Hepatosomatic indices, K = condition factor, MDA = malondialdehyde.

[4]Below detection limit

[5]Not significant

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

relation to the average weights of about 560 g pre-challenge. The concentration of Cx muscle of the Cx only treatments were significantly higher when compared to total carotenoid concentrations (Ax + Cx = $Carot_{Total}$) in fish fed diets with other carotenoid inclusion types. In alignment with this, previous studies reported improved deposition of dietary Cx into muscle towards Ax in salmon (0.8 – 0.9 kg), when diets contained only one carotenoid (Buttle et al., 2001; Page and Davies, 2006). When both carotenoids were included simultaneously at varying ratios, the relationship between muscle deposition and dietary supplementation was linear, when the deposition efficiency of the respective carotenoid was taken into account (Baker et al., 2002; Buttle et al., 2001). The average concentration of Cx in fish fed the mixed carotenoid diets in the current study were only 36% of Cx concentration in flesh of the Cx only fed fish, which is much lower than what would be expected at a linear relationship. This suggested that efficiency in Cx deposition in fish fed mixed carotenoid diets was inhibited by Ax at some stage during absorption from the digestive tract, blood transport or binding to the muscle. This contradiction to previous studies might be explained by higher dietary $Carot_{Total}$ in our study (100 mg/kg), compared to these studies, where diets with $Carot_{Total}$ ranging from 45 and 71 mg/kg were tested (Baker et al., 2002; Buttle et al., 2001). The deposition of Ax in flesh of Atlantic salmon over the entire seawater grow-out phase (0.1 – 3.2 kg) levelled off when dietary Ax concentration exceeded 60 mg/kg (Torrissen et al., 1995). However, to the best of my knowledge this is the first study reporting an inhibition of Cx deposition into the muscle due to presence of Ax in salmon. Feeding diets with varying ratios of Ax and Cx in a previous study led to the inhibition in blood absorption of both carotenoids, also with higher prominence of this negative interaction for Ax (Kiessling et al., 2003). This may indicate that the inhibition in Cx deposition in the mixed carotenoid treatments was due to inhibition of Cx absorption from the intestinal tract. Nevertheless, due to small fish sizes tested and improved carotenoid deposition with increasing weights in salmon (Torrissen et al., 1995), our results still need to be confirmed in large fish.

Some areas on the fillet surface visually appeared substantially paler than other areas post-challenge. This applied particularly to the anterior/dorsal and ventral fillet areas, which is well in line with visual appearance of commercial salmon fillets of harvest size salmon after an elevated temperature event (unpublished data). Image analysis revealed significantly decreased a* values in the anterior dorsal fillet area (Figure 2.1), whereas a* values on a reference area on the dorsal part of the NQC remained unaffected post-challenge (Figure 2.2).

These results showed that conditions chosen in the challenge phase were suitable as an experimental *in vivo* model to replicate *in situ* carotenoid depletion in Atlantic salmon after an elongated period of elevated temperature and starvation.

In contrast to the localized reduction of a* by image analysis, chemically analysed carotenoid concentrations in white muscle (from the whole fillet) were not changed post-challenge. This suggested that the level of localised carotenoid depletion may not have been detectable in whole fillet homogenates. Further, carotenoids are associated with proteins in salmon white muscle (Matthews et al., 2006). The significant increase in concentration of CP in white muscle post-challenge (Table 2.3) may therefore have additionally masked a reduction in carotenoid concentrations.

2.4.3 Effect of α-tocopherol and oxidative stress on pigment depletion

The dietary requirement of A-toc in Atlantic salmon is about 50 mg/kg (Hamre, 2011), showing that the concentrations chosen in our experiment were high. Increasing the dietary A-toc concentration led to significantly higher A-toc concentration in muscle after the loading-phase (Table 2.3), which is in line with previous studies (Bjerkeng et al., 1999; Faizan et al., 2013). A period of elevated OS was associated with decreased A-toc concentrations in salmon flesh (Nordgarden et al., 2003). Due to the cessation of A-toc supply at starvation and an anticipated increase of OS due to elevated temperature, we anticipated a pronounced drop in A-toc concentrations in muscle post-challenge. Surprisingly, the A-toc concentrations in muscle were substantially increased post-challenge (Table 2.3). Tocopherols in salmon are found in high concentrations in adipose tissue and the liver, and A-toc in particular shows high affinity to the liver, especially with increasing dietary A-toc concentration (Faizan et al., 2013; Hamre and Lie, 1997). The liver also serves

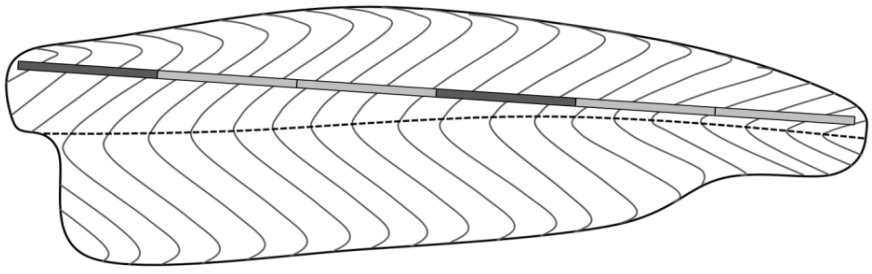

Figure 2.1 Image analysis.
A transect line with a pixel width of 32 pixels on the dorsal fillet half was divided into six equally long areas. Areas1 and 4 (in dark grey from left to right) were compared.

as a lipid depot in salmon (Forsberg, 1997). The significant decrease in weights, K and HSI in combination with significantly increased CP concentrations in muscle post-challenge (Table 2.3) may indicate that lipid was predominantly metabolised for energy during this phase. The concentrations of tocopherols are most likely regulated by lipid turnover (Hamre and Lie, 1997), indicating that the use of lipids for energy led to mobilisation of tocopherols. In contrast to γ and δ-tocopherol, circulating A-toc is not excreted to higher amounts via bile (Hamre et al., 1998). It was suggested that an A-toc binding protein in salmon liver with a high affinity to A-toc prevents the excretion of large amounts of A-toc and stimulates incorporation of retained A-toc to very low density lipoprotein for subsequent excretion into the blood stream (Hamre et al., 1998; Hamre and Lie, 1997). Liver-retained A-toc could be deposited into muscle, which could explain the significant increase in A-toc concentrations in flesh post-challenge. Dietary A-toc concentration did not affect carotenoid concentrations in muscle (Table 2.3), or a* values on the fillet areas tested (Figure 2.2).

Reactive oxygen species (ROS) are inevitably produced in aerobic organisms as a metabolic side product and are kept in steady state ROS concentrations (Lushchak, 2011). Increasing oxidative pressure (ROS production), leads to OS if ROS exceed these steady state

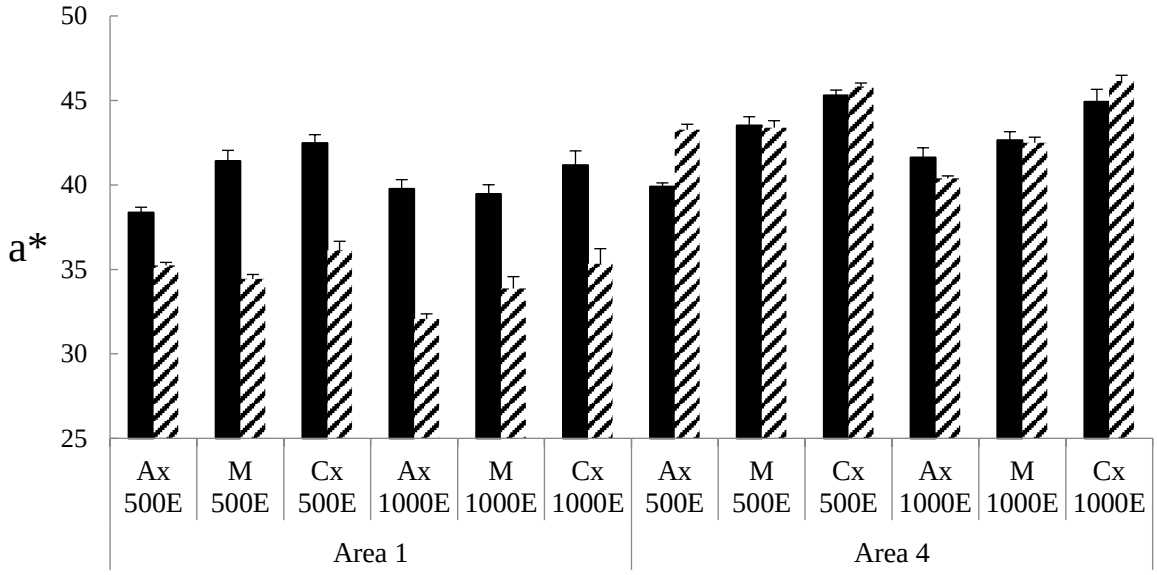

Figure 2.2 Redness chromaticities (a* values) on 2 fillet areas (areas 1 and 4, shown in Figure 2.1) of Atlantic salmon post-smolt fed diets with varying carotenoid inclusion types and α-tocopherol concentrations for 80 d (pre-challenge, solid bars) and a subsequent phase of starvation at elevated temperature (post-challenge, striped bars), respectively.
The diets were supplemented with either 100 mg/kg astaxanthin (Ax), 100 mg/kg canthaxanthin (Cx) or a mix of 50 mg/kg Ax and 50 mg/kg Cx (M); at 500 and 1000 mg/kg α- tocopherol (500E, 1000E), respectively. Dietary carotenoid inclusion type significantly affected a* values on both areas ($p \leq 0.05$ in area 1, $p \leq 0.0001$ in area 4, respectively). Redness chromaticities on area 1 were significantly decreased ($p \leq 0.0001$) post-challenge, whereas a* values on area 4 were not affected.

concentrations, leading to damage of cellular constituents such as lipid peroxidation. MDA is an end-product of lipid peroxidation and therefore an OS-indices (Lushchak, 2011). *In vivo* examination of salmon muscle that contained either Ax, A-toc or both, showed that the lowest MDA concentrations were found in muscle containing Ax and A-toc, which indicated an antioxidant synergism between these substances (Bell et al., 2000). However, this was evoked by deliberate deficiency of either one or both antioxidants (Bell et al., 2000), representing an unlikely scenario when diets with a balanced antioxidant composition are fed, as expected in commercial salmon farming. In the current study, MDA concentrations in white muscle were not affected by dietary A-toc concentration, indicating that the increase of A-toc concentration from 500 to 1000 mg/kg was not beneficial in the prevention of OS *in vivo* under the conditions tested. Moreover, the MDA concentrations in muscle indicated a lower level of OS post-challenge. The concentrations of hepatic glutathiones reinforced this inference. GSH can react with ROS leading to the conversion of two GSH molecules into one GSSG molecule (Lushchak, 2011), which can be reversed enzymatically (Bagnyukova et al., 2007). Both forms of glutathione were significantly higher pre- compared to post-challenge (Table 2.4). While higher GSH pre-challenge could have been due to enzymatic conversion of GSSG to GSH, the increased tGSH concentration pre-challenge showed that *de novo* synthesis of GSH explained this, likely as a response to increased oxidative pressure at this time. This was also in line with increased values in hepatic OSI pre-challenge. The reduction of a* occurred in the challenge-phase despite decreased OS post-challenge, which indicated that the carotenoid depletion observed was not due to the metabolic use of carotenoids as antioxidants.

It was surprising that OS was lower-post challenge, as an increase in temperature leads to increased metabolism activity in ectotherms, leading to increasing oxygen consumption and ultimately ROS production (Lushchak, 2011). Forsberg (1997) observed that average daily O_2 consumption in salmon held at 8.5°C more than doubled from starved fish to fish fed a daily rate of 0.6% of body weight. Another study tested oxygen consumption in Tasmanian salmon at 14, 18 and 22°C and found that the plateau metabolic rate was increased by only one third in the 22°C group when compared to the 14°C group (Barnes et al., 2011). Hence, elevated OS pre-challenge was likely due to increased metabolic activity associated with feeding, digestion and tissue growth in the loading phase. However, it appears conceivable that elevated OS may lead to a reduction of pigment carotenoid concentrations when salmon feed at elevated temperature.

2.4.4 Effect of challenge on product quality

The concentration of A-toc in muscle is an important feature for fillet shelf-life due to its important role in the prevention of post-slaughter peroxidation of highly unsaturated FA (HUFA), particularly in species with a high total lipid concentration like salmon (Hamre, 2011). Feeding diets with low A-toc concentrations in combination with high concentration of HUFA led to significantly increased rancid flavour in salmon fillet (Waagbø et al., 1993). The increased concentration of A-toc in muscle post-challenge may therefore indicate that the shelf life of fillets from salmon that underwent an elongated phase of elevated temperature and starvation may not be reduced. Visually, a smaller proportion of the fillet surface area was affected by pigment depletion in our study compared to severe cases *in situ* following temperature extremes. Hence, when salmon are exposed to more challenging conditions, it is likely that pigment depletion is exacerbated compared to the current study. Further, heterogeneous pigmentation on rainbow trout fillets led to lower visual acceptance by a sensory panel (Yanar et al., 2007) and causes significant product commercial downgrade in salmon products (T. Fox-Smith, pers. communication), underlining the significance of uniform fillet pigmentation for consumer acceptance and thus, marketability.

Table 2.4 Glutathione concentrations (µmol/g) and oxidative stress index (OSI) in livers of post-smolt salmon fed diets with varying carotenoid inclusion types and α-tocopherol concentrations for 80 d (pre-challenge) and a subsequent phase of starvation at elevated temperature (post-challenge), respectively

		GSH[2]	GSSG	Total GSH[3]	OSI[4]
Pre-challenge	[1]Ax500E	2.98 ± 0.151	2.42 ± 0.101	7.83 ± 0.342	60.8 ± 0.42
	M500E	3.16 ± 0.198	2.82 ± 0.417	8.81 ± 0.994	63.0 ± 2.34
	Cx500E	3.42 ± 0.288	2.93 ± 0.263	9.27 ± 0.797	62.8 ± 1.06
	Ax1000E	3.03 ± 0.200	2.42 ± 0.262	7.87 ± 0.678	60.6 ± 1.83
	M1000E	3.03 ± 0.067	2.88 ± 0.331	8.80 ± 0.678	63.6 ± 3.28
	Cx1000E	2.74 ± 0.110	2.35 ± 0.407	7.45 ± 0.914	60.8 ± 3.39
Post-challenge	Ax500E	2.55 ± 0.073	1.81 ± 0.173	6.17 ± 0.390	56.2 ± 3.45
	M500E	2.56 ± 0.199	1.74 ± 0.093	5.77 ± 0.543	57.0 ± 1.87
	Cx500E	2.55 ± 0.186	1.95 ± 0.229	6.44 ± 0.592	59.3 ± 2.02
	Ax1000E	2.70 ± 0.158	1.89 ± 0.138	6.48 ± 0.366	57.6 ± 1.70
	M1000E	2.77 ± 0.153	1.86 ± 0.259	6.50 ± 0.600	55.7 ± 3.06
	Cx1000E	2.35 ± 0.075	1.77 ± 0.065	5.89 ± 0.199	58.4 ± 0.49
3 Way-ANOVA					
Sampling time		***	***	***	*
α -tocopherol concentration		N.s.[5]	N.s	N.s	N.s
Carotenoid inclusion		N.s	N.s	N.s	N.s

[1]Diets were supplemented with either 100 mg/kg astaxanthin (Ax), 100 mg/kg canthaxanthin (Cx) or a mix of 50 mg/kg Ax and 50 mg/kg Cx (M); at 500 and 1000 mg/kg α-tocopherol (500E, 1000E), respectively
[2]GSH = Glutathione reduced, GSSG = Glutathione oxidised, [3]GSH + 2 GSSG
[4](2 GSSG / (GSH + 2 GSSG)) × 100 [5]Not significant *$p \leq 0.05$, ***$p \leq 0.001$

2.5. Summary and conclusion

A four-week phase of elevated temperature and starvation-challenge led to area specific pigment depletion on the fillet surface of post-smolt salmon, which however did not significantly affect the carotenoid concentration in the whole fillet. This occurred regardless of dietary carotenoid inclusion type and α-tocopherol concentrations fed prior to challenge under the conditions tested. The concentration of α-tocopherol in white muscle was increased and oxidative stress indices were decreased post-challenge, indicating that pigment depletion was not due to elevated oxidative stress.

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CHAPTER 3

Heterogeneous astaxanthin deposition in fillet of Atlantic salmon post-smolt at elevated temperature is not affected by dietary oil blend and not associated with the metabolic conversion of astaxanthin to idoxanthin, or oxidative stress

3.1 Abstract

Salmon producers in Tasmania experienced reduced pigmentation quality around the summer period in recent years. The study tested the effects of temperature (elevated temperature; ET, 19.5°C and control; CT, 15°C), Long chain polyunsaturated (LC-PUFA) content (diets 1 2, containing, 16.6 and 6.7% LC-PUFA of total FA, respectively) on the deposition of astaxanthin (Ax) in two fillet cuts (anterior/dorsal cut; ADC and dorsal Norwegian quality cut; dNQC) in post-smolt salmon. The metabolic conversion of Ax into idoxanthin (Ix) and oxidative stress (OS) were examined as potential underlying biological processes that may affect Ax deposition. Both diets contained about 50 mg/kg Ax and fish were fed to satiation until initial weights were doubled at the two temperatures. The concentration of Ax in white muscle was higher at ET and higher in the dNQC cut at ET, whereas Ax concentration between the fillet cuts were similar in the CT treatments. The factor diet affected lipid composition (concentrations of total lipid, triglycerides, phospholipids and FA-composition in triglycerides and phospholipids), but not the concentration of Ax in white muscle. The concentration of the OS-indices malondialdehyde in white muscle was not affected by diet or temperature and corresponded poorly with the concentration of Ax. Further, there was no idoxanthin in white muscle. When Ax was expressed per unit of crude protein in white muscle, the concentrations of Ax in crude protein was higher in the dNQC at ET. In summary, ET led to higher deposition of Ax in white muscle of the dNQC, and Ax concentration was not affected by dietary FA composition. There was no indication that OS and the metabolic conversion of Ax into Ix did to affect Ax concentrations under the conditions tested. The differences in Ax concentration per unit of crude protein at ET may have suggested differences in the affinity of myofibrillar white muscle proteins to bind pigment carotenoids.

3.2 Introduction

Pigmentation quality is among the most important criteria in salmon products and higher red colour intensity increases the willingness of consumers to pay premium (Alfnes et al., 2006). The red colour of salmon flesh is a result of pigment carotenoids like astaxanthin (Ax) deposited, and the red colour intensity shows high correlation to the concentration of pigment carotenoids (Buttle et al., 2001; Christiansen et al., 1995c). Seasonal changes in carotenoid concentrations in salmon flesh occur in marine salmon farming (Refsgaard et al., 1998; Torrissen et al., 1995) and seasonal changes in temperature may explain this phenomenon

(Refsgaard et al., 1998). In some marine farms in Tasmania, summer is associated with periods of elevated temperature (> 20°C, ET), leading to reduced pigmentation quality in farmed Atlantic salmon in sea water. This is characterised by reduced red colour intensity and increasing pigmentation heterogeneity on the fillet surface of individual fish. It was therefore hypothesised that deteriorated pigmentation quality in salmon at ET is due to decreased and more heterogeneous pigment deposition along the fillet.

Carotenoids are lipophilic and it is well established that increasing concentration of dietary total lipid can improve the efficiency of carotenoid deposition in salmonids (Bjerkeng et al., 1999a; Nickell and Bromage, 1998; Olsen et al., 2005). Moreover, differences in the carotenoid deposition efficiency were also found between salmon fed diets with the same total lipid concentration, but with different fatty acid (FA) compositions. Reduced carotenoid deposition efficiency was thereby often associated with an increasing substitution of dietary fish oil by plant oils (Regost et al., 2004; Rørå et al., 2005a; Rørå et al., 2005b), or by the use of fish oil sources with lower amounts of polyunsaturated long-chain fatty acids (LC-PUFA) (Bjerkeng et al., 1999a). However, the mechanisms involved seem not well understood and reduced pigmentation in salmon is not always observed when dietary LC-PUFA concentration is decreased (Sissener et al., 2016). Despite possible effects of dietary FA composition and temperature on pigment deposition, we are not aware of a study that tested if temperature and diets with different FA composition interact to influence carotenoid deposition efficiency in salmon.

It is well established that both, dietary FA composition and temperature affect muscle FA composition in salmon (Jobling and Bendiksen, 2003; Miller et al., 2006; Turchini et al., 2009). The FA composition in muscle is relevant for the oxidative stress (OS) susceptibility, where higher amounts of long chain LC-PUFA deposited, which are prone to oxidation, led to an increase in OS in salmon tissues (Kjær et al., 2008a; Østbye et al., 2011; Rørå et al., 2005b). Ax is a highly potent antioxidant and periods of increased OS were associated with reduced Ax concentrations in salmon flesh (Nordgarden et al., 2003). Even though increasing temperature usually increases the OS level in fish (Lushchak, 2011), a recent study showed that post-smolt salmon fed at an ideal growing temperature (15°C) generated higher levels of OS when compared to fish starved at an elevated temperature (19.5°C), which indicated that pigment depletion was not due to OS under the conditions tested. However, we also stressed that the OS level may be higher in fish feeding at elevated temperature, which could then affect the concentration of carotenoids in muscle (Grünenwald et al., *In preparation*).

Another factor that affects the concentrations of pigment carotenoids in salmon is their metabolic conversion into colourless products. The proportion of idoxanthin (Ix), the first metabolic product of Ax in salmon, can be considerable to the carotenoid pool in flesh of salmon fed diets with Ax. (Schiedt et al., 1989; Schiedt et al., 1988). However, whether ambient temperature may affect the metabolic conversion of carotenoids remains to be determined.

Despite globally increasing sea temperatures, there is a lack of knowledge as to how the deposition of pigment carotenoids in salmon is altered at elevated temperature. The current study tested the effects of temperature (control; CT; 15°C vs elevated; ET; 19.5°C) and dietary LC-PUFA concentration (diets 1 and 2 with 16.6 and 6.7% LC-PUFA of total FA, respectively) on the Ax deposition in two fillet cuts (anterior/dorsal cut; ADC and the dorsal part of the Norwegian quality cut, dNQC). It was thereby tested whether OS or the metabolic conversion of Ax into Ix are relevant metabolic processes that may affect Ax deposition under the conditions tested.

3.3 Materials and methods

3.3.1 Diets

Diets were manufactured by vacuum-coating two oil blends either containing fish oil (Diet 1, D1) or canola oil (Diet 2, D2) as the main oil source onto the same basal kernel material (Skretting, Cambridge, Australia) in order to create two diets with varying LC-PUFA contents (Table 3.1). Kernel used was of 4 mm diameter and was produced by cooking extrusion (Skretting, Cambridge, Australia). In the vacuum-coating step, the preheated (45°C) oil blends were poured on the kernel (190.5 g oil/ 1000g kernel). Then, the mixture was then mingled by hand inside a bucket and exposed to vacuum for about two minutes before the atmospheric pressure was restored swiftly to let the kernel absorb the oil. After an hour of resting at room temperature to give the kernel time to absorb remaining surface oil, diets were filled in plastic bags and kept in a cooled storage room (2-4°C), until fed to the fish.

Table 3.1 Kernel ingredients, oil blend compositions, fatty acid (FA) composition, chemical composition and astaxanthin (Ax) concentration in the experimental diets

Poultry meal	25.1	
Fish meal	20.0	
Feather meal	10.0	
Wheat whole	5.0	
Meat meal	9.2	
Wheat gluten	3.8	
Soy protein concentrate	2.5	
Lupins dehulled	2.0	
Blood meal	2.0	
Minerals, vitamin and amino acid premix	1.2	
Astaxanthin (10 %)	0.1	
Oil blend (composition shown below)	19.1	
	Diet 1	Diet 2
Fish oil		
g/100 g oil blend	71.3	28.8
g/100 g diet	13.6	5.5
Poultry oil		
g/100 g oil blend	14.9	7.1
g/100 g diet	2.8	1.4
Canola oil		
g/100 g oil blend	13.8	64.1
g/100 g diet	2.6	12.2
FA composition (g/100 g)		
14:0	7.3	3.7
∑15:0	0.6	0.3
16:0	26.4	24.3
17:0	0.5	0.4
18:0	6.1	6.3
20:0	0.5	0.3
∑Saturated FA[1]	41.6	35.5
∑16:1	9.3	6.2
18:1n9	20.4	38.1
18:1n7	2.9	2.4
∑20:1	0.4	0.4
∑Monounsaturated FA	33.0	47.1
16:4n3	1.2	0.5
18:4n3	1.0	0.3
20:4n3	0.4	0.2
20:5n3	8.8	3.3
22:5n3	0.8	0.3
22:6n3	5.1	2.2
∑n3	17.2	6.7
16:3n6	0.3	0.1
18:2n6	5.6	9.7
20:3n6	0.1	0.1
20:4n6	1.2	0.5
22:5n6	0.2	0.1
∑n6[2]	7.7	10.6
∑LC-PUFA[3]	16.6	6.7
Chemical composition (g/100 g)		
Moisture	8.9	8.5
Crude protein	49.2	49.1
Total lipid	20.9	21.7
Ash	9.31	9.74
Ax concentration (mg/kg)	56.4	47.1

[1]Includes: 19:0. [2]includes: 18:3n6 and 20:2n6.
[3] Long chain polyunsaturated FA, includes: 20:2n6

3.3.2 Fish husbandry and experimental design

The experiment was conducted at the University of Tasmania's (UTAS) aquaculture research facility (IMAS Launceston, Tasmania). The UTAS Animal Ethics Committee in accordance with the "Australian code for the care and use of animals for the scientific purposes" approved the care and use of fish for this experiment (approval number A0014015). Female, diploid Atlantic salmon smolt (~ 160g, n = 520, Petuna, Cressy, Tasmania) were smoltified under constant light in collective recirculation tanks, before transferred into an experimental recirculating aquaculture system (RAS) (16 x 350L tanks) three weeks before start of the experiment. The RAS was equipped with solids filtration, biological filtration, foam fractionation, UV disinfection and temperature control. During smoltification and acclimatisation to the RAS, fish were fed grower diets only containing Ax as the only pigment carotenoid (Skretting, Cambridge, Australia). The fish stock was redistributed over the RAS two days before start of the experiment (n = 17 fish/tank). Water quality parameters were maintained with safe limits likewise for the trial duration ($NH_4/NH_3 \leq 1$ mg/L, $NO_2 < 2$ mg/L), dissolved oxygen saturation 80-90%, system water exchange 20%/day. The salinity in the RAS was incrementally increased to 24‰ within three weeks after start of the experiment. Fish were fed to apparent satiation twice daily and uneaten feed was recovered for feed intake determination. The temperature in the CT treatment tanks located in one system line (8 tanks) was kept at 15.2°C, and the temperature in one system line was incrementally elevated over one week to 19.4°C (ET). Each diet was fed to quadruplicate tanks at each temperature, leading to the four treatments: Fish oil at control temperature (D1CT), fish oil at elevated temperature (D1ET), canola oil at control temperature (D2CT) and Canola oil at elevated temperature (D2ET), respectively. The ET treatments were fed three additional days in order to obtain similar final weights as the CT treatments at the end of the experiment, leading to 53 and 56 days of feeding for the CT and ET treatments, respectively.

3.3.3 Weight check and sampling

Individual fish weights were determined when fish were redistributed over the RAS (3.3.2) and ten fish were blindly selected were sacrificed for tissue samples and initial biometric indices on this occasion as described below. Four weeks after experimental start, fish were sedated (10 µL/L clove oil) and tank bulk weights were determined in a weight check. In the final sampling, all fish were sedated (10 µL/L clove oil), individually weighed and fork

length measured, before six fish per tank were selected blindly and anaesthetised until unresponsive with clove oil (40 µL/L) and killed by a blow to the head. Then, the livers were excised, weighed, snap frozen in liquid N and kept at -80°C before analysed for glutathiones. Then, the right-hand side fillet was removed, boned, skinned and cut along the horizontal septum. White muscle of the dorsal fillet half was cut longitudinally into six equally long cuts and the first (ADC) and fourth (dNQC) fillet-cuts (Figure 3.1) from anterior to dorsal from each tank were pooled for fillet cut and homogenised with a food processor for 11 sec. Then, subsamples were frozen in liquid nitrogen and stored at -80°C until analysed for Ax, Ix, MDA, lipid classes and FA within TAG and PL. Another subsample of the pooled fillet cut muscle homogenates was frozen at -20°C until analysed for chemical composition.

3.3.4 Chemical analysis

Astaxanthin and idoxanthin in white muscle

Pre-homogenised white muscle (1 g) was mixed with 4 mL of acetone, cooled to -20°C and homogenised using Precellys standard tubes containing 2.8 mm ceramic beads (lysing kit CK28, Bertin Technologies, France), before the mix was centrifuged at 3,000 g for 5 min. For the analysis of idoxanthin, 2 µL of the acetone extract was injected into a reverse phase HPLC system (model 1200, Agilent) using a mix of water/methanol/acetonitrile (18/20/62, v/v/v) as fluid phase and a Halo C8 column (2.7 µm, 150 x 4.6 mm, Advanced Materials Technology). The flow rate was 1.5 mL/min. The instrument setup was calibrated with an all-E idoxanthin standard (DSM Nutritional Products) and it was ensured that Ix was well separated from Ax on the chromatograms. For the analysis of Ax, 2 mL of the acetone extract was evaporated under N_2-gas at 50°C, and the vials were then cooled to room temperature

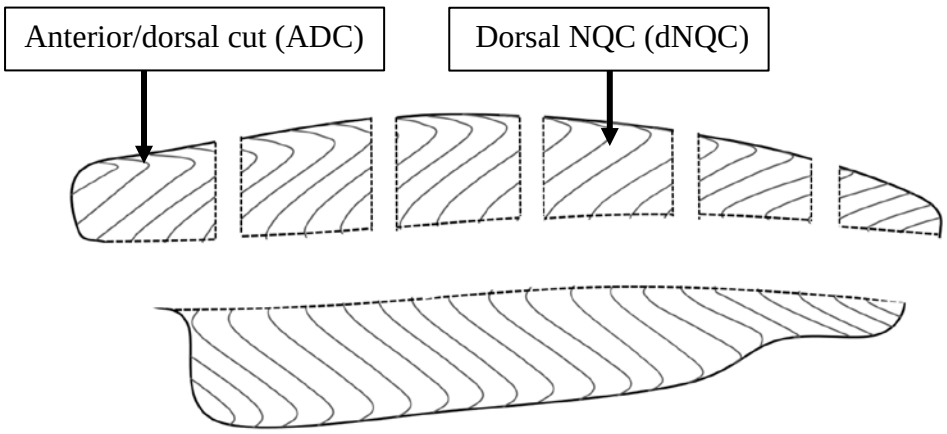

Figure 3.1 Fillet cuts sampled

before the samples were dissolved in 2 mL of a mixture of n-hexane/acetone (86:14, v/v). Then, 100 uL of sample was injected into a normal-phase HPLC via by normal Phase HPLC (model 1200, Agilent) and subsequent UV/VIS-detection at 470 nm wavelength. Analytical conditions and calibration for Ax were conducted as described previously (Schüep and Schierle, 1995), and all-E Ax standards were used for calibration (LGC, Germany).

Lipid class fractionation of white muscle lipids and fatty acid analysis

The total lipid extract (TLE) from freeze dried white muscle and diets was extracted overnight by a modified Bligh and Dyer protocol using a chloroform/methanol/water (1/2/0.8, v/v/v) overnight extraction, followed by addition of chloroform/water (1/1, v/v) the next day for phase separation. The lower phase was collected and concentrated under reduced pressure at 42°C before redissolved in chloroform (6 µL chloroform/ mg TLE). Lipid class fractionation of flesh samples TLE was conducted as described previously (Mansour et al., 2014) with some modifications. Fractionation columns were manufactured by adding 0.6-1 g of silica gel 60 (0.063-0.2 mm; Merck, NSW, Australia) into short pasteur-pipettes plugged with glass wool. Columns were then primed with n-hexane, before 50 µL of TLE solution was gently added on the surface of the column. Three elution runs per sample were conducted, consisting of 10 and 15 mL of diethyl ether in n-hexane (1/10, v/v), followed by 10 mL of methanol. The first and third elution runs contained the TAG and PL fractions, respectively, whereas the intermediate run contained TAG remainders and was withdrawn. Fraction-purity was checked in every second sample throughout the fractionation-process as described in the next section for the TLE samples. TAG and PL samples were concentrated under reduced pressure at 42°C. For FA analysis, about 1 µg sample (TAG or PL from flesh, or diet-TLE) was transferred into screw cap glass vials, then 3 mL of methanol-chloroform-HCl conc. (10/1/1, v/v/v) was added and the glass vials were sealed and heated (80°C, 1 h) for trans-methylation of FA. FA methyl esters (FAME) were extracted three times by addition of 1.8 mL of hexane/chloroform (4/1, v/v), shaking and collection of the top layer. This step was repeated two more times. Samples were then concentrated at 42°C under N_2-gas and made up to a known volume using chloroform with a known concentration of 21:0 FAME internal standard. FAME were separated using a Varian CP-3800 gas chromatograph equipped with a CP-8400 autosampler, coupled to a Brüker 300-MS triple quadrupole mass spectrometer. Stationary phase was an Agilent DB-5MS column, 30 m x 0.25 mm, with 0.25 µm phase thickness and helium was used as the carrier gas. Flow rate was 1.2 mL/min and

injector was set to 290°C. The oven began at 50°C for 1 min, then increased at 30°C/min to 150°C, then increased at 2°C/min to 250°C, then increased at 5°C/min to 320°C and held for 5 min (run time 73.3 min). Electron ionisation (EI) mass spectra were recorded in full scan mode. MS operating conditions were: source temperature 220°C, transfer line 290°C, ionising energy −70eV, scan range *m/z* 40 – 450, scan time 0.31s. FAME were identified from their mass spectra and Kovats retention indices (KI). Data was processed using MS Workstation 7.0. FA analysis in TAG and PL from white muscle were conducted in singlicate, and diet TLE was analysed in duplicate, respectively.

Lipid class analysis in white muscle

Lipid classes in white muscle were analysed in the TLE extract via thin layer chromatography and subsequent flame ionization detection. Samples (1 µL of sample solution containing 10 – 20 mg TLE/mL chloroform) were applied onto quartz rods coated with silica (solid phase) and lipid classes separated by development of the rods mobile phase, a mixture of hexane/diethyl ether/nitric acid (70/10/0.01, v/v/v) for 30 min. Then, rods were dried at 80°C for 10 min and lipid classes were analysed (IATROSCAN MK-6s, SES Analysesysteme, Bechenheim, Germany). The rods were calibrated for TAG and PL using commercial laboratory standards (Sigma Aldrich, Castle Hill, NSW, Australia). Samples were analysed in duplicate.

Glutathione and glutathione disulphide in livers

About 200 mg of each of the six livers taken from the same tank at a given sample time were transferred in a vial containing 4 mL of ice cold 1.15% (w/w) KCl solution, then homogenised for 15 s (IKA Ultra Turrax T10, basic). After homogenisation, the remaining extraction steps were conducted as described previously (Bouligand, Deroussent, Paci, Morizet & Vassal, 2006), using 50 µL of a stable isotope standard solution containing GSH (Glycine-13C2, 98%+; 15N, 96-99%; 65-70% Net; conc.: 60 µg/mL) and GSSG (Glycines-13C2, 98%+; 15N, 96-99%; 65-70% Net; conc.: 130 µg/mL, both standards were purchased from Novachem Pty Ltd, Collingwood, VIC, Australia). Extracts were stored at -80°C until analysed. The thiol GSH (and surrogate standard) were analysed as their carboxymethyl (CM) derivatives, while GSSG (and surrogate standard) were analysed as free compounds. Analyses were conducted using a Waters Acquity H-Class UPLC instrument coupled to a

Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was used, held at 35°C. The mobile phase consisted of 0.1 % (v/v) formic acid (solvent A) and acetonitrile (solvent B). Initial conditions were 100% A, decreasing to 20% A by 3.5 min, which was maintained for 0.5 min before returning to 100% A over 0.5 min and re-equilibration for 3 min. The flow rate was 0.35 mL/min. The injection volume was 10 µL. The MS/MS was operated in positive ion electrospray mode, with capillary voltage of 2.7 KV, and individual cone voltages / collision energies for each multiple reaction monitoring (MRM) transition. The desolvation temperature was 450°C.

Malondialdehyde in white muscle

Pooled and pre-homogenised white muscle tissue (about 250 mg) was transferred into a glass vial on ice containing 2 mL of 1% (v/v) sulphuric acid conc. in PBS, before 20 µL of a deuterated stable isotope surrogate standard (MDA-D2) solution was added (concentration 5.0 µg/mL). The MDA-D2 surrogate standard was derived from hydrolysis of 1,1,3,3-tetraethoxypropane; 1, 3-D2 (purity: 95%; Novachem, Collingwood, VIC, Australia) with 1% (v/v) sulphuric acid at room temperature in the dark for 2 h. This mixture was subsequently homogenised for 30 s (IKA Ultra-Turrax T10 basic), before 1 mL of homogenate-solution was transferred into a snap-cap vial and centrifuged at 10000 rpm for 30 min at 4°C. The remaining extraction steps were conducted as described previously (Faizan, Esatbeyoglu, Bayram & Rimbach, 2014), using a 1.2 mM dinitrophenylhydrazine (DNPH) in acetonitrile/formic acid (98/2, v/v) for MDA and surrogate standard derivatisation prepared fresh daily. Extracts were stored at -80°C until analysed. MDA and surrogate standard (MDA-D2) were analysed as their DNPH derivatives. UPLC-MS/MS conditions were identical to the GSH analysis method (see above), apart from the following. The mobile phase consisted of 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). Initial conditions were 60% A, held for 1 min before a gradient to 20% A by 3 min, held for 1 min, prior to returning to initial conditions and re-equilibration for 3 min.

Chemical composition of white muscle and diets

Moisture content was determined gravimetrically by freeze drying to constant weight, total lipid was analysed according to Bligh and Dyer (1959) and ash was determined by sample combustion at 600°C for 6 h. The Kjeldahl method was used to measure nitrogen (N), using

sulphuric acid (>98 %) and a copper catalyst for digestion (400°C for 2 h). Crude protein (CP) was calculated as = N × 6.25. All samples were analysed in duplicate.

Astaxanthin in diets

Astaxanthin in diets was analysed by normal phase HPLC (model 1200, Agilent) and subsequent UV/VIS-detection at 470 nm wavelength. The instruments were calibrated using all-E standards (LGC Standards GmbH, Wesel, Germany) and standard concentrations were confirmed by spectrophotometry (Cary 50, Varian). All procedures were previously described in detail (Schüep and Schierle, 1995).

3.3.5 Calculations and statistical analysis

DM feed conversion ratio (FCR) was calculated as: feed intake (g DM) / weight gain (g). Specific growth rate (SGR) expressed as % body weight gain/day was calculated as: [(Log_n final fish weight (g) – Log_n initial fish weight (g)) / feed days] × 100. DM feed intake expressed as % of BW per day was calculated as: 100 × (feed intake (g DM) / feed days) / [(initial fish weight (g) – final fish weight (g)) / 2]. Fulton's condition factor (K) was calculated as: body weight (g) / body length $(\text{cm})^3$ × 100. Hepatosomatic index (HSI) was calculated as: liver weight (g) / body weight (g) × 100. Performance parameters, biometrics and substances analysed in liver were assessed by two-factorial analysis of variance (ANOVA) with the fixed factors; diet and temperature. Parameters in fillet were assessed using three-factorial ANOVA, using the fixed factors; temperature, diet and fillet cut. Significant differences between treatment groups were determined using Tukey HSD. Except for initial weights, initial parameters are presented in tables for reference, but were not included in statistical assessments. Homogeneity of variance was ensured by visual assessment of residual plots and the significance level was set at $p \leq 0.05$ unless otherwise stated. The statistics software used was SPSS version 22.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA).

3.4 Results

3.4.1. Growth performance, morphometrics and hepatic oxidative stress

No significant differences in final weights, SGR, feed intake, DM FCR, K and HSI were observed between the treatments and there was no significant effect of temperature and diet

(Table 3.2). No significant differences between treatments, or effects of diet and temperature were detected by the end of the experiment and final concentrations of GSH, GSSG, and level of OSI, respectively (Table 3.3).

3.4.2. Astaxanthin, idoxanthin, malondialdehyde and chemical composition in white muscle

Ax concentrations were overall higher in the ET treatments, in the dNQC and in the dNQC in the ET treatments, respectively (Table 3.4) and the statistical trends were similar to the concentrations of Ax when expressed as a component of the CP fraction. There was no Ix in any of the samples. MDA concentrations were not significantly affected by the fixed factors, but there was an interaction ($p \leq 0.05$) between the factors diet and fillet cut. The concentrations of DM, total lipid and TAG were higher in the ADC and in the D2 treatments, respectively. The concentrations of PL and ash were not affected by the temperature, fillet cut and oil blend. The concentration of CP was higher in the dNQC.

3.4.3 Fatty acid composition of the phospholipid fraction in white muscle and concentrations of fatty acids from the phospholipid fraction in white muscle

The proportion of most individual FAs in the PL fraction (g FA/ 100g PL) was affected by dietary oil blend, which showed that this factor had the strongest impact on the FA composition in PL (Table 3.5). The FA composition in the PL fraction followed the dietary

Table 3.2 Effects of temperature and dietary fatty acid composition on growth performance, feed conversion and biometric indices of Atlantic salmon post-smolt.

	Initial	15°C		19.5°C	
		Diet 1	Diet 2	Diet 1	Diet 2
Initial weights (g)		212 ± 2.3	213 ± 3.1	213 ± 2.8	212 ± 1.4
Final weights (g)	-	401 ± 13.5	432 ± 10.0	421 ± 18.4	398 ± 21.2
SGR[1]	-	1.20 ± 0.071	1.34 ± 0.119	1.21 ± 0.097	1.12 ± 0.087
Feed intake DM[2]	-	1.19 ± 0.022	1.30 ± 0.059	1.21 ± 0.097	1.19 ± 0.087
Ax cons./fish (mg)[3]		12.1 ± 0.63	11.5 ± 0.72	13.3 ± 0.73	10.5 ± 1.04
FCR DM[4]	-	1.03 ± 0.031	1.02 ± 0.019	1.05 ± 0.052	1.10 ± 0.025
Condition factor	1.05	1.28 ± 0.009	1.33 ± 0.009	1.27 ± 0.022	1.29 ± 0.030
Hepatosom. indices	1.5	1.14 ± 0.045	1.07 ± 0.074	1.05 ± 0.054	1.05 ± 0.011

Values (except initial) are treatment means ± SEM, n = 4. Diet compositions are shown in table 3.2.
[1]SGR = Specific growth rate.
[2]Dry Matter feed intake expressed as % of body weight/day,
[3]Astaxanthin consumption per fish
[4]FCR = feed conversion ratio

composition. Temperature also affected the proportions of some FA in the PL fraction, whereas the proportions of only a few FA in the PL fraction were affected by fillet cut. The proportion of saturated FA (SFA) in the PL fraction was significantly higher at ET ($p \leq 0.01$), and the proportion of monounsaturated FA (MUFA) was overall higher in the D2 treatments ($p \leq 0.001$) and lower at ET ($p \leq 0.001$), respectively. The proportions of n3 FA and LC-PUFA was significantly elevated in the D1 treatments ($p \leq 0.001$) and the proportions of n6 D1 in the PL fraction was lower in the ET treatments. The latter point also applied to the FO treatments.

The concentrations (mg FA from PL/kg muscle) of MUFA, n3 FA and LC-PUFA were affected by diet, following the trends of the dietary FA composition. The concentration of MUFA from PL was decreased at ET and increased in the ADC, respectively.

3.4.4 Fatty acid composition of the triglyceride fraction in white muscle and concentrations of fatty acids from the triglyceride fraction in white muscle

In line with PL, the proportion of most individual FA in the TAG fraction (g FA/100 g TAG) was affected by diet, which showed that this factor had the strongest impact on the FA composition in TAG and this followed the trends of dietary FA composition (Table 3.6). In contrast to PL, temperature affected the proportion of only a few FA and the proportion of MUFA decreased at ET, whereas the proportions of n3 FA and LC-PUFA increased ($p \leq 0.05$). The factor fillet cut showed no impact on FA composition in TAG.

This was in contrast to the concentrations of FA from TAG in white muscle (mg FA from TAG/kg muscle). Due to the high concentration of TAG in the ADC, the concentration of every FA from TAG in muscle was higher in the ADC as well. The concentration of MUFA was higher in the D1 treatments and at CT, and the opposite was the case for n3 FA and LC-PUFA.

Table 3.3 Effects of temperature and dietary fatty acid composition on the concentrations of glutathione (GSH) and glutathione disulfide (GSSG) (µmol/g) and oxidative stress index (OSI) in post-smolt Atlantic salmon liver after doubling initial weights.

	Initial	15°C		19.5°C	
		Diet 1	Diet 2	Diet 1	Diet 2
GSH[1]	0.39	0.66 ± 0.056	0.65 ± 0.024	0.65 ± 0.024	0.70 ± 0.018
GSSG	0.55	1.74 ± 0.245	1.67 ± 0.114	1.60 ± 0.118	1.70 ± 0.125
OSI	0.73	0.83 ± 0.009	0.83 ± 0.005	0.83 ± 0.009	0.82 ± 0.020

Values (except initial) are treatment means ± SEM, n = 4. Diet compositions are shown in table 3.2.
[1]GSH = Glutathione reduced, GSSG = Glutathione oxidised, OSI = oxidative stress index = GSSG / (GSH × 2) GSSG

Table 3.4 Effects of temperature (T), dietary fatty acid composition (D) and fillet cut (C) on concentrations of astaxanthin (Ax, mg/kg), malondialdehyde (MDA, nmol/g), chemical composition, triglycerides (TAG) and phospholipids (PL) (g/100g) in post-smolt Atlantic salmon white muscle

			15°C				19.5°C				Three Way ANOVA			
	Initial		Diet 1		Diet 2		Diet 1		Diet 2		T	D	C	[2]Int.
	[1]ADC	dNQC	ADC	dNQC	ADC	dNQC	ADC	dNQC	ADC	dNQC				
Ax	0.25	0.66	[abc]1.81 ±0.08	[a]1.73 ± 0.23	[ab]1.77 ± 0.09	[ab]1.77 ± 0.12	[ab]1.78 ± 0.12	[bc]2.39 ± 0.12	[abc]1.89 ± 0.12	[c]2.44 ± 0.15	**	N.s.	**	T × C**
Ax in CP[3]	1.24	3.30	8.97 ±0.42	8.42 ± 1.13	8.89 ± 0.41	8.68 ± 0.54	8.81 ± 0.53	[bc]11.5 ± 0.69	9.49 ± 0.57	[c]11.8 ± 0.62	**	N.s.	*	T × C**
MDA	14.2	13.9	24.4 ± 1.5	28.2 ± 1.8	29.2 ± 2.3	26.7 ± 0.5	31.3 ± 1.7	27.9 ± 2.0	27.6 ± 2.0	30.6 ± 2.5	N.s.	N.s.	N.s.	D × C*
DM[4]	26.5	24.2	25.8 ± 0.28	25.5 ± 0.18	26.6 ± 0.17	26.0 ± 0.25	25.8 ± 0.34	25.6 ± 0.19	26.2 ± 0.11	25.7 ± 0.31	N.s.	*	*	N.s.
CP	20.2	20.0	20.1 ± 0.18	20.6 ± 0.18	19.9 ± 0.10	20.4 ± 0.27	20.2 ± 0.19	20.7 ± 0.22	19.9 ± 0.15	20.6 ± 0.23	N.s.	N.s.	***	N.s.
Ash			1.44 ± 0.04	1.64 ± 0.08	1.52 ± 0.10	1.52 ± 0.09	1.32 ± 0.08	1.41 ± 0.10	1.42 ± 0.07	1.48 ± 0.09	N.s.	N.s.	N.s.	N.s.
TL	3.58	3.16	[bc]3.45 ± 0.03	[ab]2.69 ± 0.07	[d]4.30 ± 0.12	[ab]3.17 ± 0.15	[abc]3.25 ± 0.30	[a]2.57 ± 0.17	[cd]3.98 ± 0.13	[ab]2.94 ± 0.19	N.s.	***	***	N.s.
TAG	2.75	2.48	[bc]2.90 ± 0.03	[cd]2.22 ± 0.07	[a]3.77 ± 0.10	[cd]2.66 ± 0.17	[bcd]2.74 ± 0.28	[d]2.09 ± 0.18	[ab]3.44 ± 0.13	[cd]2.46 ± 0.20	N.s.	***	***	N.s.
PL	0.44	0.35	0.45 ± 0.08	0.40 ± 0.02	0.43 ± 0.02	0.42 ± 0.03	0.42 ± 0.01	0.40 ± 0.03	0.45 ± 0.02	0.40 ± 0.02	N.s.	N.s.	N.s.	N.s.

Values (except initial) are treatment means ± SEM, n = 4. Means in a row with a different superscript letter are significantly different ($p \leq 0.05$), Diet compositions are shown in table 3.2.[1]ADC = anterior/dorsal cut, dNQC = dorsal Norwegian quality cut; fillet cuts shown in Figure 3.1. [2]Interactions. [3]Ax in P = Ax in white muscle (mg/kg) × (1/ (% crude protein in muscle/100)), DM =d ry matter, CP = crude protein, TL = total lipid, TAG = triglycerides, PL = phospholipids. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Initial values were not included in statistical assessment.

3.5 Discussion

3.5.1 Performance

None of the performance parameters was affected by temperature. This was in contrast with previous studies reporting deteriorated feed intake, feed conversion and growth rates in Atlantic salmon post-smolt held at 18°C compared to fish held at lower temperatures (8-14°C) for three months (Handeland et al., 2008; Kullgren et al., 2013). The ET treatments showed higher feed intake compared to the CT treatments in the first week when temperatures were incrementally elevated. After the terminal temperature was reached, feed intake takes remained higher for some days in the ET treatments, then dropped and remained lower in comparison to the CT treatments for the remaining time (Figure 3.2). It is likely that, with extended duration of the experiment, the differences in feed intake and growth performance between the temperature groups would have become significant.

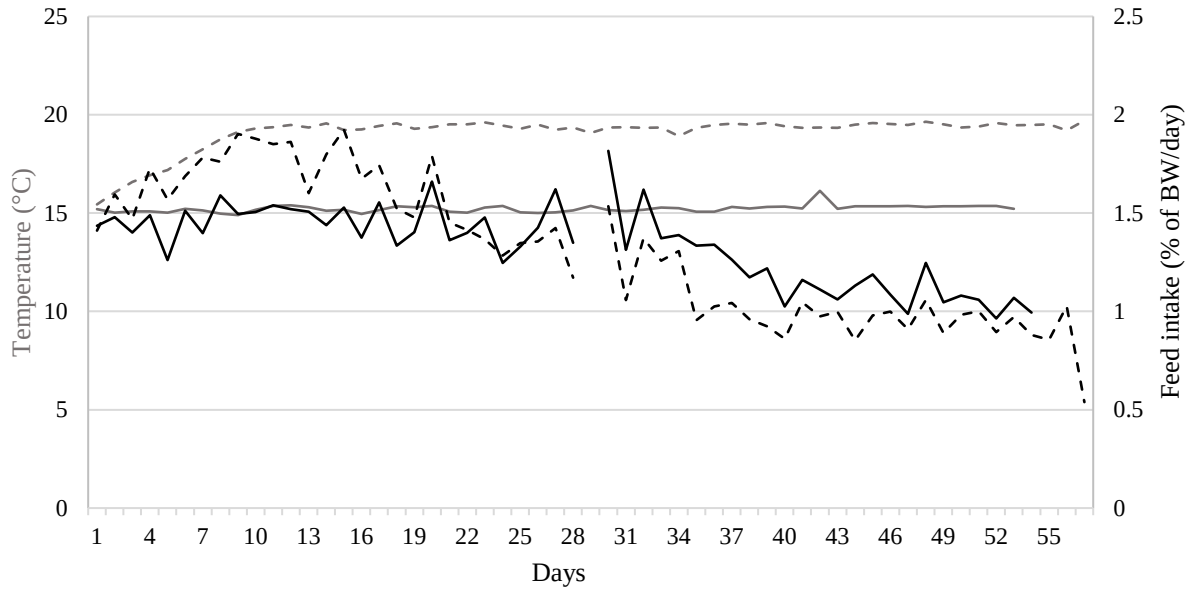

Figure 3.2 Temperatures (grey lines) and average feed intake in the elevated temperature (19.5°C, scattered lines) and low temperature (15°C, solid lines) treatments, respectively.
On day 29 a weight check was conducted and fish were not fed

Table 3.5 Effects of temperature (T), dietary fatty acid composition (D) and fillet cut (C) on fatty acid (FA) composition within the phospholipid (PL) fraction (g FA/100g PL) and concentrations of FA from the PL fraction in white muscle (mg FA/kg muscle) of Atlantic salmon post-smolt after doubling initial weights.

			15°C				19.5°C				Three Way ANOVA			
	Initial		Diet 1		Diet 2		Diet 1		Diet 2		T	D	C	[2]Int.
	[1]ADC	dNQC	ADC	dNQC	ADC	dNQC	ADC	dNQC	ADC	dNQC				
Composition														
14:0	2.1	2.0	^{ab}2.0 ± 0.2	^{ab}1.9 ± 0.1	^{ab}1.6 ± 0.1	^{ab}1.4 ± 0.1	^{b}2.2 ± 0.2	^{ab}2.0 ± 0.1	^{ab}1.4 ± 0.1	^{a}1.3 ± 0.2	[3]N.s.	***	N.s.	N.s.
∑15:0	0.3	0.3	^{ab}0.2 ± 0.0	^{ab}0.2 ± 0.0	^{ab}0.2 ± 0.0	^{ab}0.2 ± 0.0	^{c}0.3 ± 0.0	^{c}0.3 ± 0.0	^{ab}0.2 ± 0.0	^{ab}0.2 ± 0.0	N.s.	**	N.s.	T × D*
16:0	20.6	20.3	21.2 ± 2.0	22.1 ± 0.9	20.9 ± 0.5	21.9 ± 1.2	24.4 ± 0.3	24.0 ± 1.4	21.7 ± 2.2	24.8 ± 2.1	*	N.s.	N.s.	N.s.
18:0	4.8	5.4	5.8 ± 0.6	5.3 ± 0.2	5.5 ± 0.5	5.4 ± 0.2	5.1 ± 0.2	5.7 ± 0.4	6.3 ± 1.2	5.7 ± 0.5	N.s.	N.s.	N.s.	N.s.
∑SFA[4]	28.2	28.4	29.7 ± 1.6	30.1 ± 0.8	28.6 ± 0.5	29.3 ± 1.1	32.4 ± 0.3	32.4 ± 1.1	30.1 ± 1.3	32.5 ± 1.7	**	N.s.	N.s.	N.s.
∑16:1	3.4	3.4	^{ab}2.9 ± 0.4	^{ab}2.9 ± 0.3	^{ab}2.8 ± 0.2	^{ab}2.5 ± 0.2	^{ab}3.2 ± 0.4	^{ab}2.7 ± 0.1	^{ab}2.2 ± 0.2	^{ab}2.1 ± 0.3	N.s.	*	N.s.	N.s.
18:1n9	22.0	22.7	^{ab}13.7 ± 0.7	^{ab}13.6 ± 0.6	^{d}22.0 ± 0.7	^{cd}20.4 ± 0.6	^{ab}13.3 ± 0.8	^{a}12.5 ± 0.5	^{cd}19.6 ± 1.2	^{bc}17.3 ± 1.0	**	***	*	N.s.
18:1n7	2.4	2.4	2.5 ± 0.1	2.5 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	1.9 ± 0.2	N.s.	**	N.s.	N.s.
∑MUFA[5]	29.1	29.7	^{ab}19.5 ± 0.9	^{ab}19.4 ± 1.1	^{d}27.6 ± 0.8	^{cd}25.7 ± 0.6	^{ab}19.1 ± 1.2	^{a}17.8 ± 0.4	^{bcd}24.6 ± 1.4	^{abc}21.8 ± 1.2	**	***	*	N.s.
20:4n3	0.7	0.6	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	N.s.	N.s.	N.s.	N.s.
20:5n3	6.7	6.0	^{b}10.1 ± 0.6	^{b}9.7 ± 0.2	^{a}6.6 ± 0.2	^{a}7.0 ± 0.1	^{b}9.8 ± 0.2	^{b}9.6 ± 0.5	^{a}7.0 ± 0.3	^{a}7.2 ± 0.4	N.s.	***	N.s.	N.s.
22:5n3	1.9	1.7	^{bc}2.2 ± 0.2	^{c}2.3 ± 0.1	^{ab}1.6 ± 0.0	^{abc}1.7 ± 0.1	^{abc}2.1 ± 0.1	^{abc}2.1 ± 0.1	^{a}1.7 ± 0.2	^{a}1.6 ± 0.1	N.s.	***	N.s.	N.s.
22:6n3	23.2	23.9	30.4 ± 1.7	30.8 ± 1.2	25.0 ± 0.8	26.6 ± 1.2	29.1 ± 1.6	31.3 ± 0.4	27.2 ± 1.9	28.7 ± 2.3	N.s.	**	N.s.	N.s.
∑n3[6]	33.1	32.7	44.0 ± 2.3	43.8 ± 1.3	34.2 ± 0.9	36.0 ± 1.5	42.0 ± 1.5	43.9 ± 0.3	36.8 ± 2.3	38.1 ± 2.7	N.s.	***	N.s.	N.s.
18:2n6	6.7	6.3	^{ab}3.4 ± 0.1	^{a}3.3 ± 0.2	^{c}6.0 ± 0.2	^{c}5.3 ± 0.2	^{a}3.2 ± 0.2	^{a}2.8 ± 0.3	^{bc}5.1 ± 0.5	^{abc}4.4 ± 0.6	*	***	*	N.s.
20:3n6	0.3	0.3	^{abc}0.3 ± 0.0	^{b}0.3 ± 0.0	^{d}0.5 ± 0.0	^{d}0.5 ± 0.0	^{a}0.2 ± 0.0	^{a}0.2 ± 0.0	^{cd}0.4 ± 0.0	^{bcd}0.4 ± 0.0	***	***	N.s.	N.s.
20:4n6	1.3	1.4	2.0 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.0	2.1 ± 0.2	1.9 ± 0.1	1.9 ± 0.0	N.s.	*	N.s.	N.s.
22:5n6	0.3	0.2	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	*	N.s.	N.s.	N.s.
∑n6[7]	9.2	8.8	^{ab}6.4 ± 0.2	^{ab}6.4 ± 0.3	^{c}9.3 ± 0.1	^{c}8.6 ± 0.1	^{a}6.2 ± 0.2	^{a}5.7 ± 0.4	^{bc}8.3 ± 0.5	^{abc}7.4 ± 0.6	**	***	N.s.	N.s.
∑LC-PUFA[8]	34.7	34.4	46.2 ± 2.4	46.3 ± 1.3	36.9 ± 1.0	39.0 ± 1.7	44.2 ± 1.6	46.2 ± 0.5	39.2 ± 2.5	40.7 ± 2.7	N.s.	***	N.s.	N.s.
Concentration														
∑SFA[4]	123	98	134 ± 7	119 ± 7	122 ± 7	124 ± 9	137 ± 4	132 ± 13	136 ± 14	130 ± 10	N.s.	N.s.	N.s.	N.s.
18:1n9	96	79	^{ab}62.3 ± 4.5	^{a} 54.4 ± 5.2	^{c}94.0 ± 7.4	^{bc}86.0 ± 5.1	^{a}56.0 ± 3.0	^{a}50.3 ± 3.1	^{bc}88.2 ± 7.0	^{abc}68.7 ± 1.1	*	***	*	N.s.
∑MUFA[5]	127	103	^{abc}88.6 ±5.2	^{ab}77.4 ± 7.9	^{c}118 ± 8	^{bc}109 ± 7	^{ab}80.5 ± 4.5	^{a}71.4 ± 4.0	^{bc}111 ± 8	^{abc}86.5 ± 1.6	*	***	*	N.s.
∑n3[6]	145	113	200 ± 14	173 ± 7	146 ± 8	153 ± 13	177 ± 10	177 ± 11	165 ± 9.0	155 ± 21	N.s.	**	N.s.	N.s.
∑LC-PUFA[8]	152	119	210 ± 15	183 ± 7	157 ± 9	165 ± 14	187 ± 10	186 ± 11	176 ± 10	165 ± 21	N.s.	*	N.s.	N.s.

Values (except initial) are treatment means ± SEM, n = 4. Means in a row with a different superscript letter are significantly different ($p \leq 0.01$). Initial values are excluded from statistical assessment. Diet compositions are shown in table 3.2. [1]ADC = anterior dorsal fillet cut, dNQC = dorsal part of the Norwegian quality cut. Fillet cuts are displayed in Figure 3.1. [2]Interactions. [3]Not significant *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$. [4]Saturated fatty acids (FA), includes: 16:0, 17:0 and 19:0. [5]Monounsaturated FA, Includes: 20:1. [6]includes: 16:4n3 and 18:4n3. [7]Includes: 16:3n6, 18:3n6, 20:2n6 and 22:4n6. [8]Long chain polyunsaturated FA, includes: 20:2n6 and 22:4n6

Table 3.6 Effects of temperature (T), dietary fatty acid composition (D) and fillet cut (C) on fatty acid (FA) composition within the triglyceride (TAG) fraction (g FA/100g TAG) and concentrations of FA from the TAG fraction in white muscle (mg FA/kg muscle) of Atlantic salmon post-smolt after doubling initial weights

			15°C				19.5°C				Three Way ANOVA			
	Initial		Diet 1		Diet 2		Diet 1		Diet 2		T	D	C	[2]Int.
	[1]ADC	DNQC	ADC	DNQC	ADC	DNQC	ADC	DNQC	ADC	DNQC				
Composition														
14:0	3.1	2.8	^{d}5.0 ± 0.2	^{abc}4.4 ± 0.3	^{ab}3.0 ± 0.4	^{a}2.5 ± 0.3	^{cd}4.6 ± 0.1	^{cd}4.6 ± 0.5	^{a}2.5 ± 0.4	^{ab}2.9 ± 0.5	[2]N.s.	***	N.s.	N.s.
∑15:0	0.4	0.3	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	N.s.	***	N.s.	N.s.
16:0	19.2	18.7	21.2 ± 1.9	21.6 ± 0.5	21.2 ± 0.7	19.8 ± 0.4	21.5 ± 1.0	23.5 ± 0.3	20.2 ± 0.7	21.4 ± 0.9	N.s.	N.s.	N.s.	N.s.
18:0	6.1	5.6	6.2 ± 0.2	6.3 ± 0.3	5.8 ± 0.4	6.5 ± 0.4	6.0 ± 0.2	5.8 ± 0.2	6.6 ± 0.8	6.0 ± 0.8	N.s.	N.s.	N.s.	N.s.
∑SFA[4]	29.4	28.0	^{ab}33.8 ± 1.9	^{ab}33.5 ± 0.4	^{ab}31.1 ± 0.5	^{a}29.9 ± 0.4	^{ab}33.4 ± 1.0	^{b}35.2 ± 0.2	^{ab}30.4 ± 0.9	^{ab}31.2 ± 0.8	N.s.	***	N.s.	N.s.
∑16:1	5.1	5.7	^{b}8.0 ± 0.7	^{ab}7.0 ± 0.3	^{ab}5.4 ± 0.6	^{a}4.7 ± 0.4	^{ab}7.5 ± 0.3	^{ab}7.1 ± 0.5	^{a}4.6 ± 0.7	^{a}5.0 ± 0.6	N.s.	***	N.s.	N.s.
18:1n9	38.1	40.5	^{a}28.6 ± 1.4	^{ab}30.5 ± 0.4	^{c}38.5 ± 1.1	^{c}41.9 ± 0.5	^{a}28.4 ± 0.9	^{a}27.0 ± 2.7	^{bc}36.8 ± 0.6	^{bc}37.5 ± 1.8	*	***	N.s.	N.s.
18:1n7	2.9	2.9	3.3 ± 0.1	3.6 ± 0.2	3.0 ± 0.1	3.3 ± 0.1	3.2 ± 0.2	3.3 ± 0.2	2.9 ± 0.1	2.7 ± 0.2	*	**	N.s.	N.s.
∑MUFA[5]	48.9	51.7	^{ab}41.1 ± 2.2	^{ab}42.4 ± 0.5	^{bc}48.5 ± 1.3	^{c}51.8 ± 0.3	^{ab}40.5 ± 0.9	^{a}38.7 ± 3.1	^{abc}45.7 ±0.2	^{abc}46.7 ± 1.6	*	***	N.s.	N.s.
20:4n3	0.4	0.4	^{bc}0.5 ± 0.0	^{bc}0.5 ± 0.0	^{ab}0.3 ± 0.0	^{ab}0.3 ± 0.0	^{c}0.5 ± 0.0	^{abc}0.4 ± 0.0	^{abc}0.3 ± 0.0	^{a}0.3 ± 0.0	N.s.	***	N.s.	N.s.
20:5n3	2.1	1.8	^{cd}4.1 ± 0.4	^{bcd}3.7 ± 0.1	^{ab}2.1 ± 0.2	^{a}1.6 ± 0.1	^{d}4.4 ± 0.2	^{d}4.5 ± 0.8	^{abcd}2.7 ± 0.2	^{abc}2.4 ± 0.3	*	***	N.s.	N.s.
22:5n3	1.0	1.0	^{bc}1.4 ± 0.1	^{bc}1.4 ± 0.1	^{a}0.8 ± 0.0	^{a}0.7 ± 0.0	^{c}1.5 ± 0.1	^{bc}1.4 ± 0.2	^{ab}0.9 ± 0.1	^{a}0.8 ± 0.1	N.s.	***	N.s.	N.s.
22:6n3	7.6	5.6	9.9 ± 0.7	8.8 ± 0.2	6.6 ± 0.9	5.3 ± 0.9	9.7 ± 0.2	10.9 ± 2.2	9.8 ± 1.0	8.3 ± 0.6	*	**	N.s.	N.s.
∑n3[6]	11.6	9.3	^{b}16.5 ± 1.2	^{ab}15.1 ± 0.2	^{ab}10.2 ± 1.0	^{a}8.2 ± 0.8	^{b}16.9 ± 0.5	^{b}18.0 ± 3.2	^{ab}14.2 ± 1.0	^{ab}12.3 ± 1.0	*	***	N.s.	N.s.
18:2n6	8.0	9.2	^{ab}6.0 ± 0.4	^{ab}6.7 ± 0.1	^{ab}8.2 ± 0.3	^{b}8.3 ± 0.5	^{ab}6.7 ± 0.3	^{a}5.8 ± 0.2	^{ab}7.7 ± 0.8	^{ab}8.0 ± 0.5	N.s.	***	N.s.	N.s.
20:3n6	0.2	0.2	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	N.s.	*	N.s.	N.s.
20:4n6	0.5	0.4	^{b}1.0 ± 0.1	^{ab}0.9 ± 0.1	^{ab}0.7 ± 0.1	^{a}0.6 ± 0.0	^{b}1.0 ± 0.1	^{b}1.0 ± 0.1	^{ab}0.8 ± 0.1	^{ab}0.8 ± 0.0	*	***	N.s.	N.s.
22:5n6	0.1	0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	N.s.	***	N.s.	N.s.
∑n6[7]	9.5	10.5	8.0 ± 0.5	8.5 ± 0.1	9.9 ± 0.3	9.8 ± 0.5	8.8 ± 0.3	7.8 ± 0.2	9.4 ± 0.8	9.6 ± 0.6	N.s.	***	N.s.	N.s.
∑LC-PUFA[8]	12.4	10.0	^{b}17.6 ± 1.3	^{ab}16.0 ± 0.3	^{ab}11.3 ± 1.1	^{a}9.3 ± 0.9	^{b}17.9 ± 0.5	^{b}19.0 ± 3.2	^{ab}15.4 ± 1.1	^{ab}13.2 ± 0.8	*	***	N.s.	N.s.
Concentration														
∑SFA[4]	810	695	^{ab}980 ± 51	^{a}744 ± 31	^{b}1174 ± 41	^{a}798 ± 60	^{ab}910 ± 83	^{a}737 ± 67	^{ab}1049 ± 66	^{a}771 ± 69	N.s.	*	***	N.s.
18:1n9	1050	1005	^{ab}829 ± 46	^{a}676 ± 26	^{d}1449 ± 45	^{bcd}1116 ±66	^{ab}784 ± 108	^{a}575 ± 100	^{cd}1265 ± 34	^{abc}924 ± 87	*	***	***	N.s.
∑MUFA[5]	1348	1282	^{abc}1193 ±74	^{ab}941 ± 32	^{d}1826 ± 38	^{bcd}1381 ±88	^{abc}1115±143	^{a}820 ± 127	^{cd}1575 ± 53	^{abc}1150±104	*	***	***	N.s.
∑n3[6]	319	231	^{bc}479 ± 34	^{abc}334 ± 9	^{abc}386 ± 47	^{a}215 ± 13	^{bc}460 ± 38	^{abc}363 ± 47	^{c}487 ± 20	^{ab}302 ± 28	*	*	***	N.s.
∑LC-PUFA[8]	343	248	^{bc}511 ± 36	^{abc}354 ± 5	^{abc}427 ± 49	^{a}244 ± 13	^{bc}487 ± 41	^{abc}383 ± 46	^{c}525 ± 23	^{ab}325 ± 27	N.s.	*	***	N.s.

Values (except initial) are treatment means ± SEM, n = 4. Means in a row with a different superscript letter are significantly different ($p \leq 0.01$). Initial values are excluded from statistical assessment. Diet compositions are shown in table 3.2. [1]ADC = anterior dorsal fillet cut, dNQC = dorsal part of the Norwegian quality cut. Fillet cuts are displayed in Figure 3.1. [2]Interactions. [3]Not significant *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$. [4]Saturated fatty acids (FA), includes: 16:0, 17:0 and 19:0. [5]Monounsaturated FA, Includes: 20:1. [6]includes: 16:4n3 and 18:4n3. [7]Includes: 16:3n6, 18:3n6, 20:2n6 and 22:4n6. [8]Long chain polyunsaturated FA, includes: 20:2n6 and 22:4n6

3.5.2 Astaxanthin concentrations in diets, white muscle, relationship to concentrations of total lipid and crude protein

The dietary Ax concentration were about 16.5% lower in D1, which was unintended and difficult to explain, since the Ax was mixed into the kernel mix, and the same kernel material was used as the basis for both diets. Nevertheless, when the amounts of Ax consumed were calculated based on diet Ax concentration and feed intake data, there were no significant differences between the treatments, and no significant effects of diet or temperature ($p > 0.05$, Table 3.2). Hence, the differences in dietary Ax concentration did not lead to differences in the Ax consumption in this relatively short study. The choice of fillet cuts sampled was based on a recent study, where loss of redness chromaticity (a*) indicated a decrease in the pigment carotenoid concentration on the anterior/dorsal fillet area after four week starvation at ET (19.5°C). In contrast, the a*-values on the dorsal part of the NQC of the same fish was unaffected (Grünenwald et al., *In preparation*). In most studies that tested pigment carotenoid concentrations or a*-values between different fillet cuts in Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and Arctic charr (*Salvelinus alpinus*), the carotenoid concentrations, or a*-values were higher in caudal areas when compared to dorsal and anterior areas (Christiansen and Wallace, 1988; March and MacMillan, 1996; Nickell and Bromage, 1998; Olsen and Mortensen, 1997; Refsgaard et al., 1998; Skjervold et al., 2001). One study reported no differences in Ax concentration between transverse fillet cuts in harvest size (2.5 – 5 kg) Atlantic salmon (Bell et al., 1998). In the current study, there were no differences in Ax concentration between the ADC and the dNQC in the CT treatments. Due to deteriorated pigmentation quality at elevated temperatures observed at Tasmania during summer, we anticipated lower Ax concentrations in the ET treatments. It was therefore surprising that the opposite was the case. Ingested Ax is available for flesh deposition after entering circulation. This follows its digestion and passage through the gut wall (Storebakken and Goswami, 1996). Blood carotenoid concentrations in salmon increase with increasing dietary concentration up to carotenoid-specific thresholds (Kiessling et al., 2003). This could be due to deteriorated carotenoid digestibility when the dietary inclusion exceeds this threshold, but metabolic conversion during gut transit can also affect the amount of carotenoids entering circulation (White et al., 2003; Ytrestøyl et al., 2006). The dietary Ax concentrations between the D1 and D2 were similar and the total amount of carotenoids consumed were not significantly different between the temperature groups. The apparent digestibility coefficient

of Ax in Atlantic salmon was significantly increased at 12°C when compared to 8°C (Ytrestøyl et al., 2005), which showed that apparent digestibility coefficient of Ax can be affected by temperature. However, if higher digestibility of Ax caused the higher muscle concentration in the ET treatments in the current study, it is unclear why the Ax concentration was only higher in the dNQC . Arctic charr (*Salvelinus alpinus*) fed six diets containing Ax concentrations ranging from 0 to 192 mg/kg, showed significant increase in fillet a*-values up to dietary Ax concentration of 70 mg/kg (Olsen and Mortensen, 1997). This probably indicated that the concentration of Ax in circulation was increased with increasing dietary concentrations up to 70 mg/kg. However, significant differences in a*-values between an anterior and a dorsal fillet area was observed at each dietary Ax concentration (Olsen and Mortensen, 1997). This may indicate that heterogeneous pigmentation in salmonids is not affected by the availability of carotenoids for flesh deposition. We therefore suggest that higher deposition of Ax in the dNQC cut at ET was not due to increased Ax availability. Higher concentration of total lipid is sometimes associated with higher concentrations of pigment carotenoids in salmon muscle (Sissener et al., 2016; Torrissen et al., 1995). However, this association was not found in another study (Bell et al., 1998). In the current study, the concentration of total lipid showed a negative relationship to concentration of Ax (Figure 3.3). Carotenoids are attached to myofibrillar proteins in the actin myosin complex in salmon white muscle (Chacon-Ordonez et al., 2016). Hence the increase of CP, and therefore possibly also myofibrillar proteins may have explained a significant, linear relationship between the concentrations of CP and Ax (Figure 3.3). There was also a negative relationship between the concentrations of total lipid and CP in white muscle ($R^2 = 0.409$, $p \leq 0.001$, not shown). This may have indicated that the negative relationship between the concentrations of total lipid and Ax was an effect of CP dilution, rather than a causal, negative impact in the deposition of total lipid on the deposition of Ax. Due these findings, and the increased concentration of CP in the dNQC, it seemed that differences in CP concentration between the fillet cuts may have contributed to differences in Ax concentration. However, when the concentration of Ax was expressed per unit of CP, the effect of fillet cut on Ax concentration was still significant, indicating that this was not the case. It therefore seems more likely that differences in the affinity of myofibrillar proteins to bind Ax in white muscle between the fillet cuts may have caused the differences in Ax concentrations between the fillet cuts at ET.

3.5.3 Lipid composition in white muscle and metabolic conversion from astaxanthin to idoxanthin

The relationship between concentrations of total lipid and TAG was highly significant, whereas the relationship between total lipid and PL was not significant (Figure 3.4). This showed that higher deposition of TAG, not PL affected the concentration of total lipid. The concentration of TAG was higher in the D2 treatments. This was in line with higher concentrations of TAG in liver and intestine of salmon when fish oil sources was replaced by soybean oil (Jordal et al., 2007; Ruyter et al., 2006). The proportions of LC-PUFA in TAG and PL (g/100 g lipid class) were higher in the D1 treatments in the current study, particularly 20:5n3 (Eicosapentaenoic acid, EPA) and 22:6n3 (Docosahexaenoic acid, DHA). The proportion of EPA in TAG in muscle of the D1 treatments was about 40% higher compared to the D2 treatments. TAG are the main constituent of adipocytes and EPA possesses hypolipidemic properties that lead to a reduced total lipid concentration in Atlantic salmon hepatocytes (Kjær et al., 2008b). Hence, differences in the proportion of EPA in hepatocytes may have caused reduced total lipid concentration in the D1 treatments in the current study.

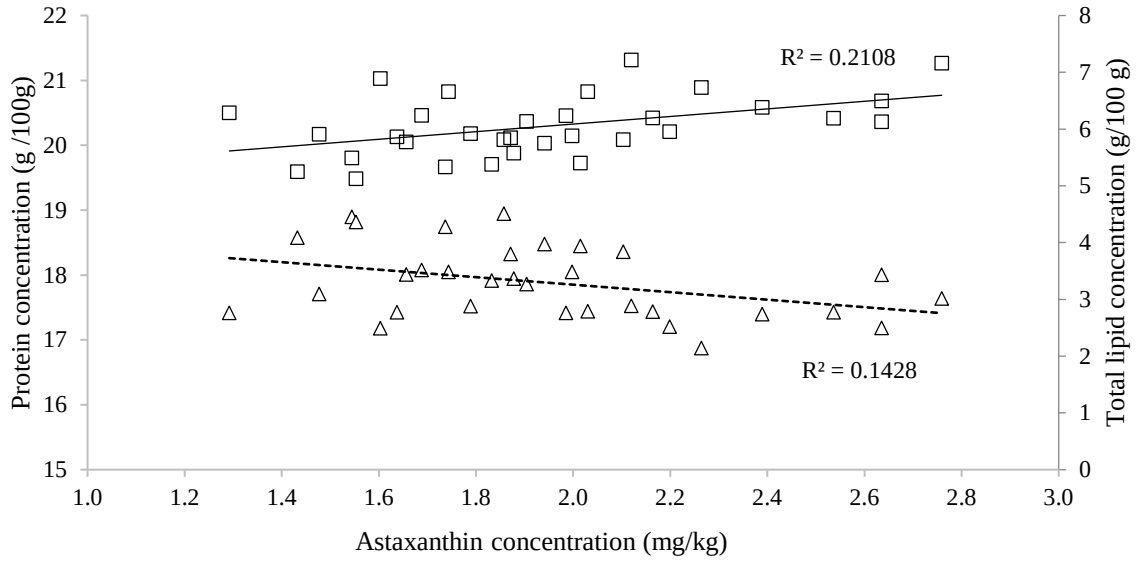

Figure 3.3 Relationship between astaxanthin (Ax) concentration and concentrations of crude protein (CP) (solid line, squares) and total lipid (TL) (scattered line, triangles) in white muscle of post-smolt salmon, respectively. Increasing CP concentration showed a positive relationship with concentrations of Ax ($p \leq 0.05$) and the opposite was the case between the concentrations of Ax and TL ($p \leq 0.05$), respectively.

In line with previous studies (Jobling and Bendiksen, 2003; Ruyter et al., 2006) the factor diet predominantly affected FA composition in TAG and PL and temperature showed some impact, especially in the PL fraction. This is an effect of cell membrane fluidity adaption (Jobling and Bendiksen, 2003; Miller et al., 2006). Minor differences exist in total lipid FA composition between different fillet areas in salmon (Refsgaard et al., 1998). To my knowledge, this is the first study to show this for FA composition in TAG and PL also. Due to significant differences in Ax concentration between the fillet cuts at ET without major differences in the FA composition within TAG and PL between the fillet cuts tested, I suggest that the differential deposition of FA between fillet cuts is not associated to differences in carotenoid pigments between different fillet cuts in salmon.

A positive correlation of fillet Ix concentration and the dietary concentration of SFA may have indicated that SFA may regulate the activity of enzymes responsible for the transformation of Ax to Ix (Bjerkeng et al., 1999b). In the current study, the concentration of SFA in D1was about 15% higher compared to D2. However, no Ix was found in any of the muscle samples. Metabolic conversion of pigment carotenoids into their first metabolic product is high in Norwegian strains of Atlantic salmon when fish are small and the conversion decreases with increasing fish size (Aas et al., 1999; Ytrestøyl et al., 2004). Due to the relatively small fish sizes by the end of the current experiment, the absence of Ix was surprising. Significantly higher concentrations in Ix were found in farmed, compared to wild Atlantic salmon of similar sizes (Schiedt et al., 1989) and the heritability coefficient of

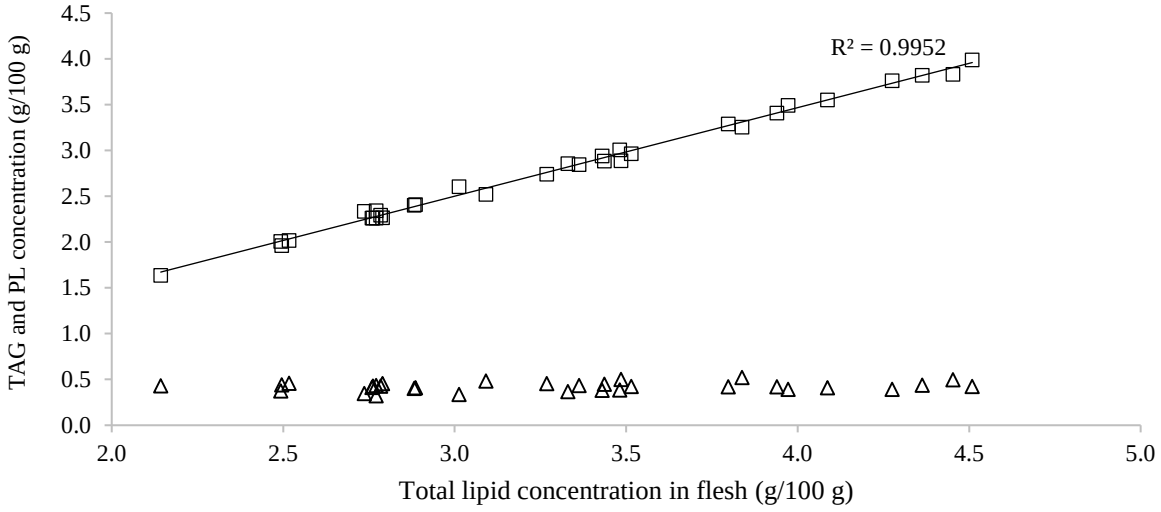

Figure 3.4 Relationships between total lipid (TL) concentration and concentrations of TAG (squares) and PL (triangles) in white muscle of salmon post-smolt.
Concentrations of TAG showed a significant relationship with TL ($p \leq 0.001$), whereas the relationship between concentrations of PL and TL was not significant ($p > 0.05$).

muscle Ix concentration is 0.4 (Bjerkeng et al., 1996) and therefore relatively high. The Tasmanian strain of Atlantic salmon originates from the same river in Nova Scotia, from where it was shipped between 1963-1965 and has been genetically isolated since this time (Ward et al., 1994). The fact that no Ix was found could therefore indicate that the lack of genetic diversity and breeding efforts in the Tasmanian population led to a genotype which is now predisposed for low transformation of Ax to Ix. Nevertheless, the fact that no Ix was found indicates that, under the conditions tested, metabolic conversion from Ax to Ix localised in muscle did not explain the differences in Ax concentration observed.

3.5.4. Lipid composition and temperature on the oxidative stress level, effect on astaxanthin concentration

LC-PUFA are highly susceptible to peroxidation due to their high number of double bonds (Shahidi and Zhong, 2010). In biological systems oxidation of LC-PUFA mainly occurs due to increasing amounts of reactive oxygen species which are produced mainly as a product of aerobic metabolism and lead to OS if the level of reactive oxygen species is not kept within a steady state level (Lushchak, 2011). reactive oxygen species can than lead to oxidation of cellular constituents like peroxidation of FA and MDA is one end-product of lipid peroxidation (Lushchak, 2011). Despite the significantly higher concentration of LC-PUFA from the TAG fraction in muscle in the ADC, the concentration of MDA was not affected by fillet cut. FA highly susceptible to oxidation such as DHA were found to be less susceptible to oxidation when attached to the *sn*-2 position within TAG (Shahidi and Zhong, 2010). In chicken meat about 90% of thiobarbituric acid reactive substances were located in the PL fraction (Pikul et al., 1984). FA deposited in PL are believed to be more susceptible to oxidation due greater proximity to the catalytic site of oxidative enzymes and other factors discussed previously (Shahidi and Zhong, 2010). The concentration of LC-PUFA from the PL fraction in white muscle was not significantly different between the fillet cuts, which may explain why despite the pronounced differences in total lipid concentration between the fillet cuts, the concentrations of MDA were not different.

Increasing temperature increases the metabolic activity in ectotherms, which can lead to increased reactive oxygen species production and thus, OS (Lushchak, 2011). However, neither concentrations of MDA in muscle, or the hepatic concentrations of GSH, GSSH and the oxidative stress index were affected by temperature. The level of OS in Atlantic salmon post-smolt feeding at 15°C was higher compared to salmon starving at ET (19.5). This

showed that metabolic processes associated to feeding outweighed the effect of elevated temperature in the generation of OS under the conditions tested (Grünenwald et al., *In preparation*). The reduced feed intake in the ET treatments during the last five weeks may therefore have contributed to the absence of elevated OS at ET in the current study. We therefore recommend for future studies to standardise the feed rates when OS is examined in teleosts. The concentration of MDA poorly corresponded with Ax concentration, indicating that the differences in Ax observed were not due to differences in the level of OS.

3.5.5 The effect of elevated temperature on pigmentation quality

The higher Ax concentration at ET may appear as a positive feature, since high carotenoid concentrations are generally desirable for salmon products. However, heterogeneous fillet pigmentation can have the opposite effect on consumer acceptability (Yanar et al., 2007). We therefore suggest that the pigmentation quality observed at ET would be rather undesirable under commercial conditions. Furthermore, the ADC, which showed lower concentration of Ax at ET, was affected by pigment depletion in contrast to another fillet area when fish were starved at 19.5°C (Grünenwald et al., *In preparation*). A higher proportion of salmon showed empty gastrointestinal tracts when held at 19°C under experimental conditions (Hevrøy et al., 2012). Asymmetry in feed intake between individuals was also associated to the summer period in Tasmania in commercial salmon farms in sea water. This was indicated by either very full or entirely empty gastrointestinal tracts in individual fish sampled for quality control (unpublished data). Hence, the more heterogeneous pigmentation of salmon fillets after a period at ET when fish are still feeding may be even exacerbated, when temperatures continue to rise and fish cease feeding.

3.6 Summary and conclusion

After post-smolt salmon doubled initial weights at 15 and 19.5°C higher concentration of astaxanthin in white muscle were found at the elevated temperature. However, significant differences in concentrations of astaxanthin were also found between an anterior/dorsal-, and the dorsal NQC cut at elevated temperature. The concentrations of astaxanthin were not affected by dietary long chain polyunsaturated fatty acid content and were not associated with indices of oxidative stress or the metabolic conversion of astaxanthin into idoxanthin, respectively. However, there were significant differences in the concentration of astaxanthin per unit of crude protein between the fillet cuts at 19.5°C. This may have indicated

differences in the affinity of myofibrillar muscle proteins between these fillet cuts to bind astaxanthin.

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CHAPTER 4

Lipid fluxes and changes in astaxanthin concentration in post-smolt Atlantic salmon (*Salmo salar* L.) muscle during starvation at different temperatures; is a switch in lipid metabolism involved in pigment depletion?

4.1 Abstract

Pigment carotenoid depletion from Atlantic salmon fillet during phases of high temperature and voluntary fasting is a major commercial issue. We tested if differences in lipid composition influence pigment depletion in salmon white muscle starving at different temperatures. In order to manipulate lipid composition in muscle prior to starvation, salmon post-smolt were fed two diets containing either fish oil, or canola oil as the main lipid source (Diets 1 and 2, respectively) at a control (CT, 15.2°C) and an elevated temperature (ET, 19.4°C) until fish doubled their initial weights. Fish were then starved at similar temperatures as they were fed (MT, 15.3°C and ET, 20.8°C) for four weeks. White muscle tissue from the anterior dorsal cut (ADC) and the dorsal part of the NQC (dNQC) examined for changes in concentrations of astaxanthin (Ax), total lipid, triglycerides (TAG), phospholipids (PL) and fatty acid (FA) composition within each of these major lipid classes. Changes in Ax concentration were not affected by diet fed. At ET, fish showed a loss in Ax concentration by about 62% in the ADC and 23% in the dNQC, respectively, whereas at CT, fish lost about 24% in the ADC and the Ax concentration in the dNQC were not significantly changed. Regression analysis showed a significant effect of initial total lipid and TAG concentrations on the loss of total lipid and TAG concentration during starvation, but this was only significant in the ET treatments. Further, differences in these relationships were found between the fillet cuts, but not dietary treatments. There were similar but weaker relationships between initial PL concentration and changes in PL concentration during starvation. Changes in FA composition showed that different FA were conserved in the two lipid classes during starvation, and the effects of temperature and diet on these features were small. The relationships between changes in Ax concentration and changes in concentrations of total lipid, TAG and PL were poor. However, there were significant relationships between the decrease of Ax concentration and the decrease of some polyunsaturated FA (PUFA) within TAG (g FA/100 TAG). These PUFA are usually conserved in feeding salmon, but oxidised to higher rates when the peroxosimal instead of the mitochondrial pathway is used for β-oxidation of FA for energy generation.

In summary, we demonstrated that the concentrations of total lipid, TAG and PL in white muscle, temperature and fillet area affected the amount of muscle-stored lipids used as energy substrate during starvation. Our data may have indicated that the onset of changes in the lipid metabolism in white muscle at starvation may have been associated to the depletion of Ax.

4.2 Introduction

Salmon possess the ability to deposit relatively large amounts of pigment carotenoids into white muscle, to give the typical “reddish” colour. Pigmentation quality in salmon products is one of the most important criteria for consumers and red colour intensity directly affects the willingness of consumers to pay premium (Alfnes et al., 2006). Sea water temperatures at some Tasmanian sites of production commonly exceed 19°C for prolonged periods (Battaglene et al., 2008) and are above the ideal temperature range for Atlantic salmon production in sea water (Battaglene et al., 2008; Kullgren et al., 2013; Miller et al., 2006). On farms exposed to temperature extremes, the summer period is anecdotally associated with generally impaired pigmentation quality, characterised by reduce red colour intensity and heterogeneous pigmentation on the fillet of individuals. This is concurrent with a pronounced drop of feed intake or even cessation of feed intake (T. Fox-Smith, pers. communication).

Starvation physiology in animals is highly complex and successful starvation-strategies must enable an animal to mobilise endogenous energy reserves from carbohydrates, protein and lipids in order to carry out the basic processes of life (McCue, 2010). The endogenous energy source used thereby depends on substrate availability and aspects of functional prioritization of different tissues and organs (McCue, 2010). In Atlantic salmon (*Salmo salar* L.), the white muscle accounts for about 50 - 60% of the total body weight (Hamre and Lie, 1995; Page and Davies, 2006). With about 15% total lipid in muscle of harvest-size salmon (Refsgaard et al., 1998; Sissener et al., 2016) the total lipid concentration is considerable. Protein metabolism in white muscle decreases over time in starving teleosts (Carter and Houlihan, 2001), and muscle-deposited total lipid is an important energy substrate for immature salmon during prolonged phases of starvation (Einen et al., 1998).

Pigment carotenoids are lipophilic and are attached to myofibrillar proteins by weak non-covalent bonds in white muscle (Birkeland and Bjerkeng, 2004). Further, previous studies reported interactions between lipids and carotenoids in salmon (Bjerkeng et al., 1999; Olsen et al., 2005). We therefore hypothesised that pigment depletion at starvation may be facilitated by the mobilisation of muscle-deposited lipids for energy during starvation.

The total lipid concentration along the fillet of salmon varies considerably and some fillet areas can have a several-fold higher concentration than other fillet areas (Einen et al., 1998; Nanton et al., 2007). The belly flap is among the fillet area with the highest concentrations in total lipid and it was reported that the reduction of total lipid from this part was higher compared to the whole fillet after long term (73 d) starvation (Lie and Huse,

1992). However, higher loss of total lipid in belly flap compared to other fillet areas was not found in another study after 86 days starvation (Einen et al., 1998). This may suggest that the onset of differential total lipid mobilisation between fillet areas could be initiated by increased ambient temperature or total lipid concentration in fillet (Einen et al., 1998). If the severity of pigment depletion is affected by the reduction of lipids at starvation, then differences in lipid reduction between fillet areas may contribute to the heterogeneous pigmentation in fillets of starving salmon stock observed in the summer period *in situ*. The vast majority of total lipid in salmon muscle is deposited in the form of triglycerides (TAG) and phospholipids (PL) (Codabaccus et al., 2011). TAG are stored in adipocyte-rich myosepta and lipid droplets in white muscle cells (Nanton et al., 2007). PL are mainly found in cell membranes and fulfil important physiological functions including cell membrane fluidity. This is why fatty acids (FA) important for membrane functions are selectively deposited into the PL fraction and FA composition between TAG and PL can differ considerably (Jobling and Bendiksen, 2003; Miller et al., 2006). TAG serve mainly as the depot for FA used for energy (Henderson and Tocher, 1987), but salmon also use FA from PL for energy during prolonged phases of starvation (Næsje et al., 2006). It therefore seems plausible that mobilisation of different lipid classes at starvation could have an effect on pigment depletion. Certain FA are preferentially utilised as energy substrate in salmon (Stubhaug et al., 2007). This may indicate that the dietary oil type fed prior to starvation could affect the amounts of FA mobilised as energy substrate from these lipid classes during starvation.

The current study tested whether changes in the concentrations of total lipid, TAG or PL show a relationship to changes in Ax concentration in white muscle of salmon after four weeks of starvation. In order to test how lipid concentrations and temperature affect lipid mobilisation, two fillets cuts with different initial total lipid concentrations were examined and fish were fed and starved at a control and an elevated temperature. Further, in order to test if lipid composition in white muscle can affect the lipid source used for energy during starvation, fish were fed two diets containing different oil sources in a preconditioning-phase preceding the starvation phase. In case that lipid mobilisation from white muscle during starvation can be manipulated by lipid composition in muscle, and lipid mobilisation affects Ax depletion, summer diets with a tailored lipid composition may be a tool to reduce pigment depletion in salmon exposed to elevated temperatures.

4.3 Materials and methods

4.3.1 Fish husbandry and experimental design

The experiment was conducted at the University of Tasmania's (UTAS) aquaculture research facility (IMAS Launceston, Tasmania). The UTAS Animal Ethics Committee in accordance with the "Australian code for the care and use of animals for the scientific purposes" approved the care and use of fish for this experiment (approval number A0014015). The average control temperature (CT) was 15.2°C (± 0.42 SD) and the average elevated temperature (ET) was 19.4°C (± 0.26 SD) after initial heat increment over two weeks. In the starvation phase, the average temperature in the ET treatments increased negligibly to 20.8ºC due to poorer performance of the heat control unit. Two diets were used to manipulate the lipid composition of white muscle in the preconditioning phase and contained either fish oil (Diet 1, D1) or canola oil (Diet 2, D2) as the main oil source (Table 4.1). The two oil blends were added onto batches of the same 4 mm kernel material (Skretting, Cambridge, TAS) by vacuum coating. The full sinking diets contained about 50 mg/kg Ax. There were four treatments: Fish oil at control temperature (D1CT), fish oil at elevated temperature (D1ET), canola oil at control temperature (D2CT) and Canola oil at elevated temperature (D2ET), respectively (n = 4 tanks/treatment).

4.3.2 Preconditioning phase

Atlantic salmon post-smolt (212 g ± 46 SD) were distributed into an experimental recirculating aquaculture system (RAS) (16 x 350L tanks) equipped with solids filtration, biological filtration, foam fractionation, UV disinfection and temperature control. Initial water temperature was 15.2°C and salt content 20‰ (salinity was incrementally increased to 24‰ within two weeks after start of the preconditioning phase). The temperature in the CT treatment tanks located in one system line (8 tanks) was kept at 15.2°C, and the temperature in one system line was incrementally elevated over seven days to 19.4°C (ET). Each of the two experimental diets were hand-fed to quadruplicate tanks at both temperatures in the morning and afternoon to apparent satiation. The ET treatments were fed three additional days in order to obtain similar final weights as the CT treatments at the end of the preconditioning phase, leading to 53 and 56 days of feeding for the CT and ET treatments, respectively.

Table 4.1 Ingredient-, oil blend-, fatty acid- and chemical compositions, and astaxanthin concentration in the experimental diets.

Ingredients (g/100 g)		
Poultry meal		25.1
Fish meal		20.0
Feather meal		10.0
Wheat whole		5.0
Meat meal		9.2
Wheat gluten		3.8
Soy protein concentrate		2.5
Lupins dehulled		2.0
Blood meal		2.0
Minerals, vitamin and amino acid premix		1.2
Astaxanthin (10%)		0.1
Oil blend		19.1
	Diet 1	Diet 2
Fish oil		
% of oil blend	71.3	28.8
% of diet	13.6	5.5
Poultry oil		
% of oil blend	14.9	7.1
% of diet	2.8	1.4
Canola oil		
% of oil blend	13.8	64.1
% of diet	2.6	12.2
Fatty acid composition (g/100 g lipid)		
14:0	7.3	3.7
16:0	26.4	24.3
18:0	6.1	6.3
∑Saturated fatty acids[1]	41.6	35.5
∑16:1	9.3	6.2
18:1n9	20.4	38.1
∑MUFA[2]	33.0	47.1
20:5n3	8.8	3.3
22:5n3	0.8	0.3
22:6n3	5.1	2.2
∑n3[3]	17.2	6.7
18:2 n6	5.6	9.7
20:4n6	1.2	0.5
∑n6[4]	7.7	10.6
Chemical composition (g/100 g)		
Moisture	8.9	8.5
Crude protein	49.2	49.1
Total lipid	20.9	21.7
Crude Ash	9.31	9.74
Astaxanthin (mg/kg)	56.4	47.1

[1]Contains: 15:0, 17:0, 19:0 and 20:0. [2]Contains: 18:1b-7, 20:1n7, 20:1n9 and 20:1. [3]Contains: 16:4n3, 18:4n3 and 20:4n3. [4]Contains: 16:3n6, 18:3n6, 20:2n6, 20:3n6 and 22:5n6.

4.3.3 Starvation phase

After the preconditioning phase, fish were starved for four weeks in their tanks at similar temperatures (see section 4.2.1) as the respective treatments were held at during the preconditioning phase.

4.3.4 Sampling

Fish were sampled at the end of the preconditioning phase (pre-starvation) and at the end of the starvation phase (post-starvation), respectively. All fish were anaesthetised in their respective tanks, individually weighed and fork length measured, before six fish per tank were selected blindly and anaesthetised until unresponsive with clove oil (40 µL/L) and killed by a blow to the head. Then, the livers were excised and weighed, the right-hand side fillet was removed, boned, skinned and cut along the horizontal septum. White muscle of the dorsal fillet half was cut longitudinally into six equally long cuts and the first (anterior dorsal cut; ADC) and fourth (dorsal part of the NQC; dNQC) fillet-cuts from anterior to dorsal from each tank were pooled for fillet cut and homogenised with a food processor for 11 sec. Then, subsamples were frozen in liquid nitrogen and stored at -80°C until analysed for Ax, lipid classes and FA within TAG and PL. Another subsample of the pooled fillet cut-homogenates was frozen at -20°C until analysed for chemical composition.

4.3.5 Chemical analysis

Astaxanthin in white muscle

Pre-homogenised white muscle (1 g) was mixed with 4 mL of acetone, cooled to -20°C and homogenised using Precellys standard tubes containing 2.8 mm ceramic beads (lysing kit CK28, Bertin Technologies, France), before the mix was centrifuged at 3,000 g for 5 min. Then, 2 mL of the supernatant was evaporated under N2-gas at 50°C, and the vials were then cooled to room temperature before the samples were dissolved in 2 mL of a mixture of n-hexane/acetone (86:14, v/v). Then, 100 uL sample was injected into a normal-phase HPLC system (model 1200, Agilent) for subsequent UV/VIS-detection at 470 nm wavelength. Analytical conditions and calibration were conducted as described previously (Schüep and Schierle, 1995), and all-E Ax standards were used for calibration (LGC, Germany). All samples were analysed in duplicate.

Lipid class fractionation of white muscle and fatty acid analysis

The total lipid extract (TLE) from freeze dried muscle and diets was extracted overnight by a modified Bligh and Dyer protocol using a chloroform/methanol/water (1/2/0.8, v/v/v) overnight extraction, followed by addition of chloroform/water (1/1, v/v) the next day for phase separation. The lower phase was collected and concentrated under reduced pressure at 42°C before redissolved in chloroform (6 µL chloroform/ mg TLE). Lipid class fractionation of muscle samples TLE was conducted as described previously (Mansour et al., 2014) with some modifications. Fractionation columns were manufactured by adding 0.6-1 g of silica gel 60 (0.063-0.2 mm; Merck, NSW, Australia) into short pasteur-pipettes plugged with glass wool. Columns were then primed with n-hexane, before 50 µL of TLE solution was gently added on the surface of the column. Three elution runs per sample were conducted, consisting of 10 and 15 mL of diethyl ether in n-hexane (1/10, v/v), followed by 10 mL of methanol. The first and third elution runs contained the TAG and PL fractions, respectively, whereas the intermediate run contained TAG remainders and was withdrawn. Fraction-purity was checked in every second sample throughout the fractionation-process as described in the next section for the TLE samples. TAG and PL samples were concentrated under reduced pressure at 42°C. For FA analysis, about 1 µg sample (TAG or PL from flesh, or diet-TLE) was transferred into screw cap glass vials, then 3 mL of methanol-chloroform-HCl conc. (10/1/1, v/v/v) was added and the glass vials were sealed and heated (80°C, 1 h) for trans-methylation of FA. FA methyl esters (FAME) were extracted by addition of 1.8 mL of hexane/chloroform (4/1, v/v), shaking and collection of the top layer. This step was repeated two more times. Samples were then concentrated at 42°C under N-gas and made up to a known volume using chloroform with a known concentration of 21:0 FAME internal standard. FAME were separated using a Varian CP-3800 gas chromatograph equipped with a CP-8400 autosampler, coupled to a Brüker 300-MS triple quadrupole mass spectrometer. Stationary phase was an Agilent DB-5MS column, 30 mm x 0.25 mm, with 0.25 µm phase thickness and Helium was used as the carrier gas. Flow rate was 1.2 mL/min and injector was set to 290°C. The oven began at 50°C for 1 min, then increased at 30°C/min to 150°C, then increased at 2°C/min to 250°C, then increased at 5°C/min to 320°C and held for 5 min (run time 73.3 min). Electron ionisation (EI) mass spectra were recorded in full scan mode. MS operating conditions were: source temperature 220°C, transfer line 290°C, ionising energy −70eV, scan range *m/z* 40 – 450, scan time 0.31s. FAME were identified from their mass spectra and Kovats retention

indices. Data was processed using MS Workstation 7.0. FA TAG and PL samples white analysed as singlicates, and diet TLE were analysed in duplicate, respectively.

Lipid class analysis in white muscle

Lipid classes in white muscle were analysed in the TLE extract via thin layer chromatography and subsequent flame ionization detection. Samples (1 µL of sample solution containing 10 – 20 mg TLE/mL chloroform) were applied onto quartz rods coated with silica (solid phase) and lipid classes separated by development of the rods mobile phase, a mixture of hexane/diethyl ether/nitric acid (70/10/0.01, v/v/v) for exactly 30 min. Then, rods were dried at 80°C for 10 min and lipid classes were analysed (IATROSCAN MK-6s, SES Analysesysteme, Bechenheim / Germany). The rods were calibrated for TAG and PL using commercial laboratory standards (Sigma Aldrich, Castle Hill, NSW, Australia). All samples were analysed in duplicate.

Chemical composition of white muscle and diets

Moisture content was determined gravimetrically by freeze drying to constant weight, total lipid was analysed according to Bligh and Dyer (1959) and ash was determined by sample combustion at 600°C for 6 h. The Kjehldahl method was used to measure nitrogen (N), using sulphuric acid (>98 %) and a copper catalyst for digestion (400°C for 2 h). Crude protein (CP) was calculated as = N × 6.25. All samples were analysed in duplicate.

Astaxanthin in diets

Astaxanthin in diets was analysed by normal phase HPLC (model 1200, Agilent) and subsequent UV/VIS-detection at 470 nm wavelength. The instruments were calibrated using all-E standards (LGC Standards GmgH, Wesel, Germany) and standard concentrations were confirmed by spectrophotometry (Cary 50, Varian). All procedures were previously described in detail (Schüep and Schierle, 1995).

4.3.6 Calculations and statistical analysis

Fulton's condition factor (K) was calculated as: body weight (g) / body length $(cm)^3 \times 100$. Hepatosomatic index (HSI) was calculated as: liver weight (g) / body weight (g) × 100. Change during starvation was calculated as: final concentration – initial concentration. Tank,

or fillet cuts within tank (where stated) was the unit of statistical assessment. All results presented in tables were compared using 3-Way ANOVA to test the effects of the factors sample time, temperature and diet and Tukey HSD was used to determine treatment differences. All statistical analyses in tables was based on treatment means (± SEM), and % change-data is displayed, but not used included in the analyses. Homogeneity of variance was evaluated by examination of residual plots and fatty acid data (g/100 g lipid class) was arcsine-transformed if necessary. Linear regression analysis was used to examine various relationships. Statistics software used was SPSS version 24.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). The level of significance was set at $p \leq 0.05$ unless otherwise stated.

4.4 Results

4.4.1 Mortalities, weight, condition and hepatosomatic index

Weights, K and HSI were significantly reduced post starvation ($p \leq 0.001$) (Table 4.2). K was

Table 4.2 Weights and biometric indices of post-smolt salmon before (Pre) and after (Post) a four week phase of starvation.
Prior to starving, fish were fed diets either containing canola oil (D2) or fish oil (D1) as the main lipid source until initial weights were doubled. Fish were held either at a control (15°C, CT) or an elevated (20°C, ET) temperature, respectively

		Weight (g)	Condition	[1]HSI
Pre	D1CT	[abc]401 ± 13.5	[a]1.28 ± 0.02	[a]1.14 ± 0.05
	D2CT	[a]432 ± 10.0	[a]1.33 ± 0.00	[ab]1.07 ± 0.07
	D1ET	[ab]421 ± 18.4	[a]1.27 ± 0.02	[abc]1.05 ± 0.05
	D2ET	[abc]398 ± 21.2	[a]1.29 ± 0.03	[abc]1.05 ± 0.01
Post	D1CT	[bc]336 ± 6.7	[b]1.10 ± 0.03	[bcd]0.88 ± 0.02
	D2CT	[abc]363 ± 20.6	[b]1.15 ± 0.02	[cd]0.83 ± 0.10
	D1ET	[abc]365 ± 27.2	[b]1.15 ± 0.01	[d]0.72 ± 0.01
	D2ET	[c]331 ± 25.4	[b]1.16 ± 0.02	[d]0.75 ± 0.01
% Change during starvation	D1CT	-16.2	-14.3	-0.25
	D2CT	-16.1	-13.6	-0.24
	D1ET	-13.3	-9.4	-0.33
	D2ET	-16.8	-10.3	-0.31
	[2]*3-Way ANOVA*			
	Sample time (S)	***	***	***
	Temp (T)	N.s.	N.s.	*
	Diet (D)	N.s.	*	N.s.
	Interactions	T × D*	S × T*	N.s.

Values presented as treatment mean (n = 4) ± SEM. Values in the same column with different superscript letters are significantly different (Tukey HSD, $p \leq 0.05$). [1]Hepatodsomatic index. [2]N.s.: Not significant *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

significantly higher in the D2 treatments and was significantly higher in fish held at ET post-starvation ($p \leq 0.05$). HSI was decreased in the ET treatments ($p \leq 0.05$). One fish of the D1CT treatment died in the starvation phase (0.4% of entire population).

4.4.2 Astaxanthin concentration, chemical composition and concentration of lipid classes in white muscle

Ax concentration in muscle decreased in the ADC post starvation ($p \leq 0.001$) and was lower in the ET treatments post starvation, respectively ($p \leq 0.001$) (Table 4.3). In the dNQC the concentration of Ax was higher in the ET treatments pre-starvation ($p \leq 0.005$). The concentration of CP in the ADC was overall increased post-starvation and in the D1 treatments ($p \leq 0.05$). The concentrations of total lipid and TAG in both cuts were higher in the CT treatments ($p \leq 0.05$) and in the D2 treatments (ADC: $p \leq 0.001$, dNQC: $p \leq 0.05$).

4.4.3 Fatty acid composition in triglycerides and phospholipids

The proportions of most FA in TAG (g FA/100 g TAG) were significantly ($p \leq 0.001$) affected by diet and reflected dietary FA composition (Table 4.4). In the ADC, proportions of 20:5n3, 22:6n3 and sum n3 were reduced, whereas the proportions of 18:1n9 and monounsaturated FA (MUFA) were increased post-starvation. In the same fillet cut, the proportion of 14:0 was lower, and 20:4n6 higher in the ET treatments. In the dNQC, the proportions of 16:0 and saturated FA (SFA) in TAG was significantly ($p \leq 0.005$) lower post-starvation, whereas the proportions of 18:2n6 and sum n6 increased.

The factor diet affected the proportions of MUFA, n3 and n6 within PL (g FA/100 g PL) in both fillet cuts, reflecting the dietary composition (Table 4.5). Also in both fillet cuts, the proportions of 14:0, sum 16:1 and MUFA were reduced ($p \leq 0.001$) and proportions of 22:6n3, sum n3 and 20:4n6 increased post-starvation.

4.4.4 Relationships between initial concentrations of total lipid, triglycerides and phospholipids, and changes in concentrations of total lipid, triglycerides and phospholipids during starvation

The relationship between initial concentration total lipid and changes in total lipid concentration was not significant when all data was tested (Figure 4.1 A), but was significant ($p \leq 0.05$) for the ET

Table 4.3 Changes in the concentration of astaxanthin (Ax, mg/kg), chemical composition, triglycerides (TAG) and phospholipids (PL) (g/100 g) in muscle from two fillet cuts (anterior/dorsal cut, A; and dorsal NQC, B) of post-smolt salmon before (Pre) and after (Post) a four-week phase of starvation.
Prior to starving, fish were fed diets either containing fish oil (D1) or canola oil (D2) or as the main lipid source until initial weights were doubled. Fish were held either at a control (15°C, CT) or elevated (20°C, ET) temperature, respectively.

	A	Ax	Dry matter	Crude protein	Ash	Total lipid	TAG	PL
Pre	D1CT	^{ab}1.81 ± 0.08	25.8 ± 0.28	20.1 ± 0.12	1.44 ± 0.04	^{ab}3.45 ± 0.03	^{bc}2.90 ± 0.03	0.45 ± 0.08
	D2CT	^{ab}1.77 ± 0.09	26.6 ± 0.12	19.9 ± 0.10	1.53 ± 0.10	^{a}4.30 ± 0.12	^{a}3.77 ± 0.10	0.43 ± 0.02
	D1ET	^{ab}1.78 ± 0.12	25.8 ± 0.34	20.2 ± 0.19	1.32 ± 0.08	^{b}3.25 ± 0.30	^{c}2.74 ± 0.28	0.42 ± 0.01
	D2ET	^{a}1.89 ± 0.12	26.2 ± 0.11	19.9 ± 0.15	1.42 ± 0.07	^{ab}3.98 ± 0.13	^{abc}3.44 ± 0.13	0.45 ± 0.02
Post	D1CT	^{c}1.26 ± 0.14	26.0 ± 0.22	20.5 ± 0.17	1.34 ± 0.04	^{ab}3.71 ± 0.07	^{abc}3.13 ± 0.07	0.50 ± 0.03
	D2CT	^{bc}1.35 ± 0.09	26.3 ± 0.37	20.1 ± 0.17	1.44 ± 0.05	^{a}4.15 ± 0.37	^{ab}3.59 ± 0.33	0.45 ± 0.03
	D1ET	^{d}0.65 ± 0.05	26.1 ± 0.22	20.4 ± 0.09	1.30 ± 0.03	^{ab}3.56 ± 0.11	^{abc}3.03 ± 0.10	0.45 ± 0.01
	D2ET	^{d}0.70± 0.10	25.8 ± 0.12	20.3 ± 0.22	1.34 ± 0.05	^{b}3.22 ± 0.11	^{c}2.74 ± 0.11	0.40 ± 0.02
[1]% CH	D1CT	-30.4	0.5	1.8	-7.0	7.7	7.9	11
	D2CT	-23.7	-3.5	1.3	-6.2	-3.5	-4.7	5.1
	D1ET	-63.7	1.3	1.1	-2.6	9.5	10.5	7.2
	D2ET	-62.9	1.4	2.1	-5.8	-19.1	-20.5	-11.6
[2]*3-Way ANOVA*								
Sample time (S)		***	N.s.	*	N.s.	N.s.	N.s.	N.s.
Temp (T)		***	N.s.	N.s.	N.s.	*	*	N.s.
Diet (D)		N.s.	N.s.	*	N.s.	**	**	N.s.
Interactions		S × T***	N.s.	N.s.	N.s.	S × D*	S × D*	N.s.

	B	Astaxanthin	Dry matter	Crude Protein	Ash	Total lipid	TAG	PL
Pre	D1CT	^{bc}1.73 ± 0.23	25.5 ± 0.18	20.6 ± 0.18	1.64 ± 0.08	^{ab}2.69 ± 0.07	2.22 ± 0.07	0.40 ± 0.02
	D2CT	^{abc}1.77 ± 0.11	26.0 ± 0.25	20.4 ± 0.27	1.52 ± 0.09	^{ab}3.17 ± 0.15	2.66 ± 0.17	0.42 ± 0.03
	D1ET	^{ab}2.39 ± 0.12	25.6 ± 0.19	20.8 ± 0.19	1.41 ± 0.10	^{ab}2.57 ± 0.17	2.09 ± 0.18	0.40 ± 0.03
	D2ET	^{a}2.44 ± 0.15	25.7 ± 0.31	20.6 ± 0.23	1.48 ± 0.09	^{ab}2.94 ± 0.19	2.46 ± 0.20	0.40 ± 0.27
Post	D1CT	^{c}1.70 ± 0.10	25.4 ± 0.15	20.4 ± 0.25	1.37 ± 0.04	^{ab}2.41 ± 0.19	1.94 ± 0.18	0.39 ± 0.02
	D2CT	^{abc}2.10 ± 0.12	25.9 ± 0.42	20.7 ± 0.26	1.49 ± 0.05	^{a}3.31 ± 0.48	2.81 ± 0.45	0.41 ± 0.02
	D1ET	^{abc}1.90 ± 0.12	25.6 ± 0.22	21.0 ± 0.09	1.51 ± 0.07	^{ab}2.48 ± 0.04	2.01 ± 0.10	0.41 ± 0.03
	D2ET	^{abc}1.87 ± 0.14	25.5 ± 0.10	21.2 ± 0.09	1.54 ± 0.05	^{b}2.27 ± 0.09	1.85 ± 0.08	0.36 ± 0.04
% CH	D1CT	-1.7	-0.5	-0.6	-16.7	-10.6	-12.4	-1.9
	D2CT	18.7	-0.4	1.5	-1.5	4.2	5.6	-3.2
	D1ET	-20.5	-0.2	1.1	7.2	-3.6	-3.9	2.3
	D2ET	-26	-0.5	2.9	3.8	-22.6	-25.1	-9.9
3-Way ANOVA								
Sample time (S)		N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.
Temp (T)		**	N.s.	*	N.s.	*	*	N.s.
Diet (D)		N.s.	N.s.	N.s.	N.s.	*	*	N.s.
Interactions		S × T**	N.s.	N.s.	S × T*	N.s.	N.s.	N.s.

Values presented as treatment mean (n = 4) ± SEM. Values in the same column with different superscript letters are significantly different (Tukey HSD, $p \leq 0.05$).
[1]% change during starvation
[2]N.s.: Not significant *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

Table 4.4 Changes in fatty acid (FA) composition within triglycerides (g FA/100 g TAG) in muscle from two fillet cuts (anterior/dorsal cut, A; and dorsal NQC, B) of post-smolt salmon before (Pre) and after (Post) a four week-phase of starvation. Prior to starving, fish were fed diets either containing fish oil (D1) or canola oil (D2) as the main oil source until initial weights were doubled. Fish were held either at a control (15°C, CT) or elevated (20°C, ET) temperature, respectively.

A		14:0	16:0	∑SFA[1]	∑16:1	18:1n9	∑MUFA	20:5n3	22:6n3	∑n3	18:2 n6	20:4n6	∑n6
Pre	D1CT	^{a}5.0 ± 0.2	21.2 ± 1.9	33.8 ± 1.9	^{a}8.0 ± 0.7	^{d}28.6 ± 1.4	^{bc}41.1 ± 2.2	^{ab}4.1 ± 0.4	9.9 ± 0.7	^{a}16.6 ± 1.2	^{b}6.0 ± 0.4	^{ab}1.0 ± 0.1	^{b}8.1 ± 0.5
	D2CT	^{ab}3.1 ± 0.4	21.2 ± 0.7	31.1 ± 0.5	^{ab}5.4 ± 0.6	^{ab}38.5 ± 1.1	^{ab}48.5 ± 1.3	^{c}2.1 ± 0.2	6.6 ± 0.9	^{bc}10.2 ± 1.0	^{ab}8.2 ± 0.3	^{c}0.7 ± 0.1	^{ab}9.9 ± 0.3
	D1ET	^{a}4.6 ± 0.1	21.5 ± 1.0	33.4 ± 1.0	^{ab}7.5 ± 0.3	^{d}28.4 ± 0.9	^{c}40.5 ± 0.9	^{a}4.4 ± 0.0	9.7 ± 0.2	^{a}16.9 ± 0.5	^{b}6.7 ± 0.3	^{ab}1.0 ± 0.1	^{ab}8.8 ± 0.3
	D2ET	^{b}2.5 ± 0.4	20.2 ± 0.7	30.4 ± 0.9	^{b}4.7 ± 0.7	^{abc}36.8 ± 0.6	^{abc}45.7 ± 0.2	^{bc}2.7 ± 0.2	9.8 ± 1.0	^{abc}14.2 ± 1.0	^{ab}7.7 ± 0.8	^{abc}0.8 ± 0.1	^{ab}9.4 ± 0.8
Post	D1CT	^{a}4.7 ± 0.2	20.9 ± 0.8	32.5 ± 1.0	^{ab}7.3 ± 0.3	^{cd}30.9 ± 1.6	^{abc}42.8 ± 1.4	^{ab}3.8 ± 0.3	8.0 ± 1.0	^{ab}14.9 ± 1.3	^{ab}7.4 ± 0.4	^{abc}0.9 ± 0.0	^{ab}9.3 ± 0.8
	D2CT	^{ab}3.2 ± 0.3	19.2 ± 0.8	29.0 ± 0.9	^{ab}5.4 ± 0.4	^{a}40.1 ± 0.9	^{a}50.2 ± 0.9	^{c}2.0 ± 0.1	5.4 ± 0.6	^{c}9.2 ± 0.7	^{a}9.5 ± 0.3	^{c}0.6 ± 0.0	^{a}11.3 ± 0.3
	D1ET	^{ab}3.7 ± 0.7	21.4 ± 0.9	34.0 ± 1.9	^{ab}6.2 ± 0.9	^{bcd}32.1 ± 1.8	^{abc}43.4 ± 1.2	^{abc}3.4 ± 0.4	9.0 ± 0.7	^{abc}14.6 ± 1.0	^{b}5.9 ± 0.6	^{a}1.0 ± 0.0	^{b}7.7 ± 0.8
	D2ET	^{b}2.5 ± 0.2	20.0 ± 1.0	30.4 ± 1.7	^{b}4.8 ± 0.3	^{a}39.5 ± 1.6	^{a}48.9 ± 1.6	^{c}2.0 ± 0.2	7.2 ± 0.6	^{bc}10.7 ± 1.1	^{ab}8.3 ± 0.4	^{bc}0.7 ± 0.2	^{ab}9.8 ± 0.5
% Change during starvat.	D1CT	-6.1	-1.5	-4	-7.7	8.1	4	-7.2	-18.7	-9.8	22.5	-12.6	16.4
	D2CT	4.4	-9.6	-6.8	-1.1	4.1	12	-7.7	-17.3	-9.7	15.8	-8.9	13.4
	D1ET	-18.1	-1	1.9	-17.7	13.2	7.2	-23.4	-6.8	-13.8	-12.4	2.4	-11.8
	D2ET	-3.3	-1.2	0.1	2.7	7.5	6.8	-26.5	-26.6	-25	7.6	-10.6	4.4
[2]3-Way ANOVA	S.-time (S)	N.s.	N.s.	N.s.	N.s.	*	*	*	*	*	N.s.	N.s.	N.s.
	Temp (T)	*	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	*	N.s.	N.s.	*	N.s.
	Diet (D)	***	N.s.	***	***	***	***	***	**	***	***	***	***
	Interactions	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	S × T*	N.s.	S × D*
B		14:0	16:0	∑SFA	∑16:1	18:1n9	∑MUFA	20:5n3	22:6n3	∑n3	18:2 n6	20:4n6	∑n6
Pre	D1CT	4.4 ± 0.3	^{ab}21.6 ± 0.5	^{ab}33.5 ± 0.4	7.0 ± 0.3	^{cd}30.5 ± 0.4	^{bcd}42.4 ± 0.5	^{abc}3.7 ± 0.1	^{ab}8.8 ± 0.2	^{abc}15.1 ± 0.2	^{ab}6.7 ± 0.1	^{ab}0.9 ± 0.1	^{bc}8.5 ± 0.1
	D2CT	2.5 ± 0.3	^{b}19.8 ± 0.4	^{bcd}29.9 ± 0.4	4.7 ± 0.4	^{a}41.9 ± 0.5	^{a}51.8 ± 0.3	^{c}1.6 ± 0.1	^{b}5.3 ± 0.8	^{c}8.2 ± 0.8	^{ab}8.3 ± 0.5	^{b}0.6 ± 0.0	^{ab}9.8 ± 0.5
	D1ET	4.6 ± 0.5	^{a}23.5 ± 0.3	^{a}35.2 ± 0.2	7.2 ± 0.5	^{d}27.0 ± 2.7	^{d}38.7 ± 3.1	^{a}4.5 ± 0.8	^{a}11.0 ± 2.2	^{a}18.0 ± 3.2	^{c}5.8 ± 0.2	^{a}1.1 ± 0.1	^{c}7.8 ± 0.2
	D2ET	2.9 ± 0.5	^{ab}21.4 ± 0.9	^{bcd}31.2 ± 0.8	5.0 ± 0.6	^{abc}37.5 ± 1.7	^{abcd}46.7 ± 1.6	^{bcd}2.4 ± 0.3	^{ab}8.3 ± 0.6	^{abc}12.3 ± 1.0	^{ab}8.0 ± 0.5	^{ab}0.8 ± 0.0	^{abc}9.6 ± 0.6
Post	D1CT	4.5 ± 0.4	^{ab}21.2 ± 0.4	^{abc}32.7 ± 0.6	7.0 ± 0.4	^{cd}29.5 ± 2.0	^{cd}41.1 ± 2.0	^{ab}4.0 ± 0.5	^{ab}10.1 ± 1.1	^{ab}17.0 ± 2.1	^{ab}6.7 ± 0.3	^{a}1.0 ± 0.1	^{abc}8.8 ± 0.3
	D2CT	2.6 ± 0.3	^{b}18.2 ± 0.8	^{d}28.0 ± 0.7	4.9 ± 0.4	^{a}41.5 ± 1.1	^{ab}51.2 ± 0.9	^{cd}2.0 ± 0.1	^{ab}6.1 ± 0.3	^{bc}9.7 ± 0.5	^{a}9.0 ± 0.1	^{ab}0.7 ± 0.1	^{a}10.7 ± 0.1
	D1ET	4.2 ± 0.6	^{ab}21.1 ± 0.5	^{ab}33.3 ± 0.5	6.7 ± 0.7	^{bcd}31.2 ± 1.8	^{abcd}42.9 ± 1.5	^{abc}3.6 ± 0.3	^{ab}8.4 ± 0.2	^{abc}15.0 ± 0.8	^{ab}6.6 ± 0.1	^{a}1.0 ± 0.1	^{bc}8.5 ± 0.2
	D2ET	2.7 ± 0.2	^{b}19.1 ± 1.0	^{cd}29.1 ± 1.2	5.0 ± 0.4	^{ab}40.1 ± 1.4	^{abc}49.4 ± 1.1	^{cd}2.1 ± 0.1	^{ab}6.8 ± 0.4	^{abc}10.7 ± 0.6	^{a}8.9 ± 0.4	^{ab}0.7 ± 0.1	^{a}10.5 ± 0.3
% Change during starvat.	D1CT	2.6	-1.9	-2.5	0.3	-3.1	-3.1	7.8	15.3	12.6	0.3	16.1	3.1
	D2CT	4.1	-8.1	-15	4.1	-0.9	-1.2	19.6	14.3	18.6	7.7	20.7	8.9
	D1ET	-7.2	-10.3	-5.5	-6.2	15.4	10.8	-20	-19.1	-16.9	13	-5.8	9.3
	D2ET	-5.4	-10.6	-6.9	0.6	7	6	-12.8	-17.5	-12.8	11.5	-12.4	9.4
3-Way ANOVA	S.-time (S)	N.s.	**	**	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	*	N.s.	*
	Temp (T)	N.s.	*	*	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.
	Diet (D)	***	***	***	***	***	***	***	***	***	***	***	***
	Interactions	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	S × T*	N.s.	N.s.	N.s.	N.s.

Values presented as treatment mean (n = 4) ± SEM. Values in the same column with different superscript letters are significantly different (Tukey HSD, $p \leq 0.01$).
[1]Saturated FA, contains: 15:0, 17:0, 18:0, 19:0 and 20:0; Monounsaturated FA (∑MUFA) contains: 18:1b-7, 20:1n7, 20:1n9 and 20:1; ∑n3 contains: 16:4n3, 18:4n3, 20:4n3 and 22:5n3; ∑n6 contains: 16:3n6, 18:3n6, 20:2n6, 20:3n6 and 22:5n6. [2]S.-time: sample time, N.s.: Not significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Table 4.5 Changes in fatty acid composition within phospholipids (g FA/100 g PL) in muscle from two fillet cuts (anterior/dorsal cut, A; and dorsal NQC, B) of post-smolt salmon before (Pre) and after (Post) a four-week phase of starvation. Prior to starving, fish were fed diets either containing fish oil (D1) or canola oil (D2) as the main oil source until initial weights were doubled. Fish were held either at a control (15°C, CT) or elevated (20°C, ET) temperature, respectively.

A		14:0	16:0	∑SFA[1]	∑16:1	18:1n9	∑MUFA	20:5n3	22:6n3	∑n3	18:2 n6	20:4n6	∑n6
Pre	D1CT	^{a}2.0 ± 0.2	21.2 ± 2.0	^{ab}29.7 ± 1.6	^{ab}2.9 ± 0.4	^{cd}13.7 ± 0.7	^{bc}19.6 ± 0.9	^{a}10.1 ± 0.6	^{ab}30.5 ± 1.7	^{ab}44.0 ± 2.3	^{cde}3.4 ± 0.1	2.0 ± 0.1	^{bc}6.4 ± 0.2
	D2CT	^{ab}1.6 ± 0.1	20.9 ± 0.5	^{ab}28.6 ± 0.5	^{ab}2.8 ± 0.2	^{a}22.0 ± 0.7	^{a}27.6 ± 0.8	^{c}6.6 ± 0.2	^{b}25.0 ± 0.8	^{b}34.2 ± 0.9	^{a}6.0 ± 0.2	1.9 ± 0.1	^{a}9.3 ± 0.1
	D1ET	^{a}2.2 ± 0.2	24.4 ± 0.3	^{ab}32.4 ± 0.3	^{a}3.2 ± 0.4	^{cd}13.3 ± 0.8	^{c}19.1 ± 1.2	^{a}9.8 ± 0.2	^{ab}29.1 ± 1.6	^{ab}42.0 ± 1.6	^{cde}3.2 ± 0.2	2.0 ± 0.0	^{c}6.2 ± 0.2
	D2ET	^{ab}1.4 ± 0.1	21.7 ± 2.2	^{ab}30.1 ± 1.3	^{ab}2.2 ± 0.2	^{ab}19.6 ± 1.2	^{ab}24.6 ± 1.4	^{c}7.0 ± 0.3	^{ab}27.2 ± 1.9	^{b}36.8 ± 2.3	^{ab}5.2 ± 0.5	1.9 ± 0.1	^{ab}8.3 ± 0.5
Post	D1CT	^{ab}1.4. ± 0.1	21.2 ± 1.7	^{ab}29.0 ± 1.2	^{ab}2.3 ± 0.2	^{d}12.3 ± 0.3	^{c}17.1 ± 0.4	^{a}9.8 ± 0.2	^{a}34.4 ± 0.7	^{a}47.4 ± 0.9	^{de}3.1 ± 0.2	2.3 ± 0.2	^{bc}6.4 ± 0.3
	D2CT	^{a}0.9 ± 0.2	17.9 ± 2.9	^{b}25.6 ± 2.6	^{b}1.6 ± 0.2	^{bcd}16.6 ± 1.0	^{bc}20.7 ± 1.2	^{abc}8.2 ± 0.6	^{ab}33.7 ± 2.7	^{ab}44.8 ± 3.2	^{abc}4.8 ± 0.3	2.6 ± 0.2	^{a}8.7 ± 0.5
	D1ET	^{ab}1.5 ± 0.2	26.1 ± 0.8	^{a}34.9 ± 0.8	^{ab}2.1 ± 0.2	^{cd}12.5 ± 0.6	^{c}17.1 ± 0.6	^{ab}9.6 ± 0.4	^{ab}30.1 ± 1.0	^{ab}42.3 ± 1.0	^{e}2.5 ± 0.3	2.5 ± 0.1	^{c}5.6 ± 0.5
	D2ET	^{a}0.9 ± 0.1	23.7 ± 1.7	^{ab}31.6 ± 1.8	^{b}1.6 ± 0.1	^{bc}16.7 ± 0.1	^{bc}20.6 ± 0.2	^{bc}7.6 ± 0.2	^{ab}30.4 ± 1.1	^{ab}40.2 ± 1.5	^{bcd}4.2 ± 0.1	2.3 ± 0.1	^{abc}7.5 ± 0.3
% Change during starvat.	D1CT	-28.1	0.1	-2.1	-22.7	-10.3	-13	-3.4	13	7.6	-8.9	11.2	-0.9
	D2CT	-41.2	-14.3	-10.6	-44.7	-24.5	-25.2	24.2	34.8	31.6	-20	38.7	-5.7
	D1ET	-34.4	6.6	7.7	-33.7	-5.8	-10.7	-1.8	3.3	8	-22.7	22.1	-8.8
	D2ET	-31.8	9.2	5	-29.4	-14.9	-16.5	8.5	11.9	9.3	-19.1	22.3	-9.2
[2]*3-Way ANOVA*	S. time (S)	***	N.s	N.s.	***	***	***	N.s.	***	**	***	***	N.s.
	Temp (T)	N.s.	*	***	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	*	N.s.	**
	Diet (D)	***	N.s	*	**	***	***	***	N.s.	**	***	N.s.	***
	Interactions	N.s.	N.s	N.s.	N.s.	S × D*	S × D*	S × D*	N.s.	N.s.	N.s.	N.s.	N.s.
B		14:0	16:0	∑SFA	∑16:1	18:1n9	∑MUFA	20:5n3	22:6n3	∑n3	18:2 n6	20:4n6	∑n6
Pre	D1CT	^{ab}1.9 ± 0.1	22.1 ± 0.9	30.1 ± 0.8	^{a}2.9 ± 0.3	^{bcd}13.6 ± 0.6	^{b}19.4 ± 1.1	^{ab}9.7 ± 0.2	^{ab}30.8 ± 1.2	^{ab}43.8 ± 1.3	^{bcd}3.3 ± 0.2	^{ab}2.1 ± 0.1	^{ab}6.4 ± 0.3
	D2CT	^{abcd}1.4 ± 0.1	22.0 ± 1.1	29.3 ± 1.1	^{abc}2.5 ± 0.2	^{a}20.4 ± 0.6	^{a}25.7 ± 0.6	^{c}7.0 ± 0.1	^{b}26.6 ± 1.2	^{b}36.0 ± 1.5	^{a}5.3 ± 0.2	^{b}1.9 ± 0.1	^{a}8.6 ± 0.1
	D1ET	^{a}2.0 ± 0.1	24.0 ± 1.4	32.4 ± 1.1	^{ab}2.7 ± 0.1	^{bcd}12.5 ± 0.5	^{bc}17.8 ± 0.4	^{ab}9.6 ± 0.5	^{ab}31.3 ± 0.4	^{ab}43.4 ± 0.3	^{cd}2.8 ± 0.3	^{ab}2.1 ± 0.2	^{b}5.7 ± 0.4
	D2ET	^{bcd}1.3 ± 0.2	24.9 ± 2.1	32.5 ± 1.7	^{abc}2.1 ± 0.3	^{ab}17.3 ± 1.0	^{ab}21.8 ± 1.2	^{c}7.2 ± 0.4	^{ab}28.7 ± 2.3	^{b}38.1 ± 2.7	^{abc}4.4 ± 0.6	^{b}1.9 ± 0.0	^{ab}7.4 ± 0.6
Post	D1CT	^{abc}1.6 ± 0.1	23.5 ± 1.2	31.4 ± 1.0	^{abc}2.5 ± 0.1	^{cd}12.2 ± 0.9	^{bc}17.1 ± 1.1	^{ab}9.7 ± 0.2	^{ab}32.3 ± 0.8	^{ab}44.9 ± 0.6	^{bcd}3.3 ± 0.4	^{ab}2.2 ± 0.0	^{ab}6.4 ± 0.4
	D2CT	^{cd}1.0 ± 0.1	19.8 ± 2.3	28.6 ± 1.9	^{bc}1.7 ± 0.1	^{ab}17.3 ± 1.1	^{ab}21.5 ± 1.2	^{c}7.3 ± 0.6	^{ab}31.6 ± 2.5	^{ab}41.5 ± 3.1	^{ab}4.7 ± 0.4	^{ab}2.4 ± 0.2	^{a}8.3 ± 0.4
	D1ET	^{cd}1.0 ± 0.1	22.0 ± 2.6	29.9 ± 1.8	^{c}1.5 ± 0.1	^{d}9.7 ± 0.6	^{c}13.2 ± 0.8	^{a}11.0 ± 0.1	^{a}37.3 ± 1.5	^{a}51.0 ± 1.8	^{d}2.1 ± 0.1	^{a}2.7 ± 0.3	^{b}5.6 ± 0.5
	D2ET	^{d}0.9 ± 0.1	24.6 ± 1.1	31.9 ± 0.8	^{c}1.5 ± 0.2	^{abc}15.9 ± 1.1	^{b}19.5 ± 1.5	^{bc}7.8 ± 0.2	^{ab}31.6 ± 1.3	^{ab}41.2 ± 1.4	^{abcd}3.9 ± 0.2	^{ab}2.4 ± 0.1	^{ab}7.2 ± 0.3
% Change during starvat.	D1CT	-13.8	6.3	4.2	-13.6	-10.7	-11.7	-0.1	4.8	2.6	2	5.9	0.1
	D2CT	-25.6	-10	-2.4	-33.5	-15.3	-16.3	5.1	18.9	15.2	-11.4	23.6	-4
	D1ET	-48.3	-8.7	-7.9	-44.9	-22.7	-25.6	14.5	19.2	17.5	-24.7	27.9	-1.3
	D2ET	-28.1	-1.2	-1.8	-27	-8.1	-10.4	8	10	8.2	-11.3	25.1	-2.3
3-Way ANOVA	S. time (S)	***	N.s.	N.s.	***	**	***	*	**	**	N.s.	***	N.s.
	Temp (T)	*	N.s.	N.s.	*	**	***	N.s.	N.s.	N.s.	**	N.s.	**
	Diet (D)	***	N.s.	N.s.	**	***	***	***	*	***	***	N.s.	***
	Interactions	S × T*	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.	N.s.

Values presented as treatment mean (n = 4) ± SEM. Values in the same column with different superscript letters are significantly different (Tukey HSD, $p \leq 0.01$).
[1]Saturated FA, contains: 15:0, 17:0, 18:0, 19:0 and 20:0; Monounsaturated FA (∑MUFA) contains: 18:1b-7, 20:1n7, 20:1n9 and 20:1; ∑n3 contains: 16:4n3, 18:4n3, 20:4n3 and 22:5n3; ∑n6 contains: 16:3n6, 18:3n6, 20:2n6, 20:3n6 and 22:5n6. [2]S.-time: sample time, N.s.: Not significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

treatments and highly significant for individual fillet cuts of the ET treatments ($p \leq 0.001$), respectively (Figure 4.1 B). The relationship between initial TAG and changes in TAG during starvation was very similar to the relationship of initial total lipid and change in total lipid (Figure 4.1, C and D). Higher initial PL concentration led to higher loss of PL concentration during starvation when all data was tested, for the dNQC of the CT, both cuts of the ET, and the ADC of the ET treatments, respectively ($p \leq 0.05$) (Figure 4.2).

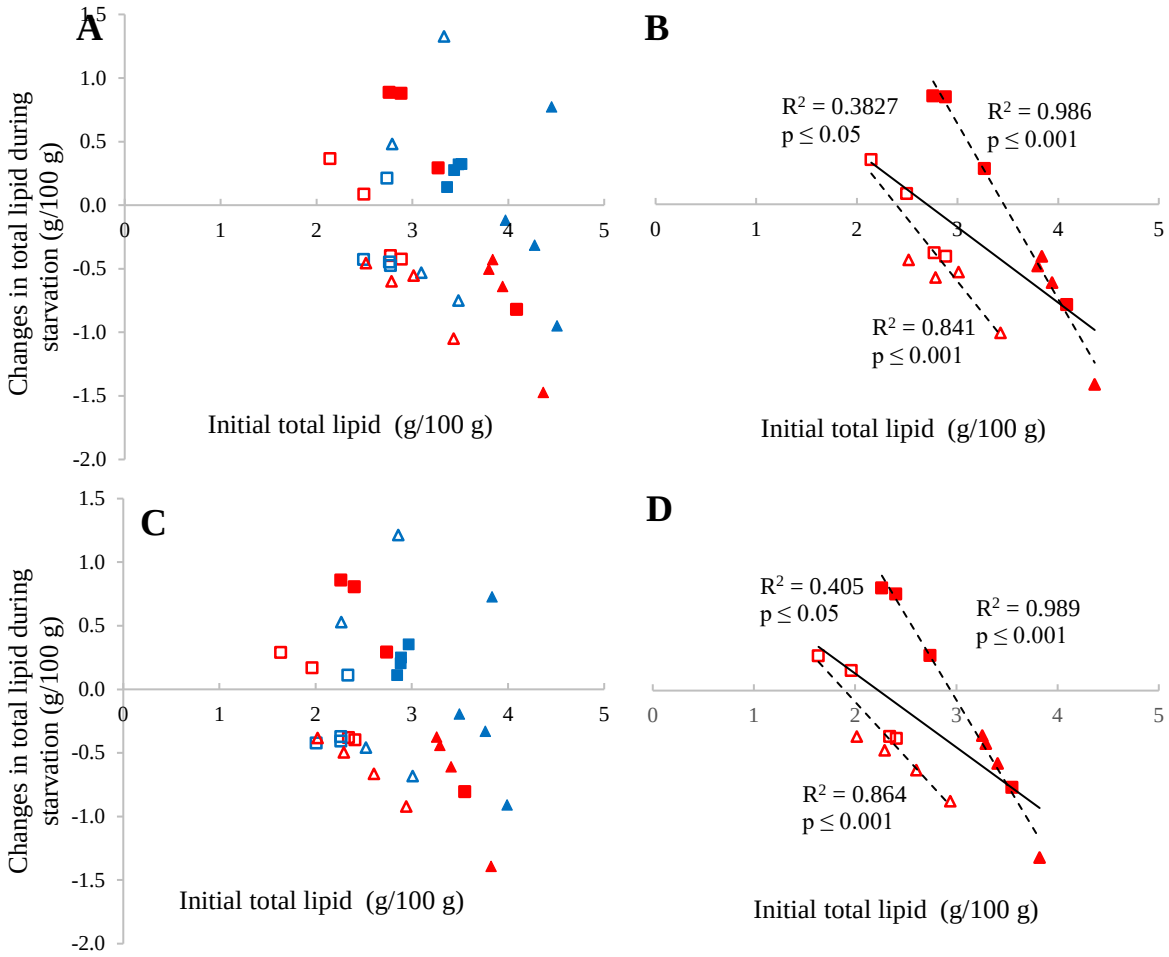

Figure 4.1 The relationship of initial total lipid concentration and changes in total lipid concentration (A and B), and the relationship of initial triglycerides (TAG) concentration (horizontal axes, g/100 g) and changes in TAG concentration (vertical axes, g/100 g) (C and D) in post-smolt salmon white muscle after a four-week phase of starvation.
Prior to starvation, fish were fed two diets containing either fish oil (squares) or canola oil (triangles) as the main oil source until initial weights were doubled. Fish were held either at control (15°C, blue) or elevated (20°C, red, ET) temperature, respectively. The anterior/dorsal fillet cut (filled marker) and dorsal NQC (open marker) were tested. Significant relationships were observed when all fillet cuts at ET were pooled (B and D, solid lines) and for the individual cuts at ET, respectively (B and D, scattered lines).

4.4.5 Relationships between changes in astaxanthin concentration and changes in concentrations of total lipid, triglycerides and phospholipids, and fatty acids within lipid class during starvation

The relationships between changes in the concentration of Ax and the concentrations of total lipid, TAG or PL were not significant when all data were tested, and after splitting the data for temperature or fillet cuts within temperature, respectively (data not shown). Further, the relationships between changes individual FA in PL (g FA/100 g PL) and changes in Ax concentration were not significant. The reduction of the polyunsaturated FA (PUFA) in TAG: 20:5n3, 22:5n3, 18:3n6, 20:3n6 and 22:5n3 (g FA/100 g TAG), as well as the sum n3 FA in TAG was in a significant relationship with the reduction of Ax ($p \leq 0.05$), whereas the inverse relationship was observed between loss of Ax and 20:0 in TAG ($p \leq 0.05$), (examples shown in Figure 4.3).

4.5 Discussion

The effects of diet, temperature and fillet cut on the deposition of lipids and Ax in white muscle were discussed in Chapter 3 in detail, using the preconditioning phase of the current experiment. Using the same fish, this chapter tested how the lipid composition in white muscle prior to starvation affected changes in lipids during a starvation phase and how these changes related to changes in Ax concentration.

4.5.1 Overview, loss of body weight and condition

The general metabolic activity in ectotherms is increased at increased temperature and increased temperature led to higher depletion of energy in Atlantic silverside (*Menidia menidia*) during starvation (Schultz and Conover, 1999). A previous study found that salmon starved at lower temperature showed increased gene-expression of enzymes involved in proteolysis (Carter et al., 2008), which may indicate that salmon preferentially used protein as energy source at low temperature. With a mean value of 23.6 kJ/g, the energy density of protein is considerably lower compared to lipids with an energy density of 39.5 kJ/g (NRC, 2011), the use of protein as energy substrate therefore requires higher amounts compared to lipid. Moreover, each unit of protein accommodates about four units of water (Dumas et al., 2007), which fortifies the loss of body weight (and volume) considerably when protein is used as energy substrate at starvation. The fact that fish starved at CT had a higher loss in K

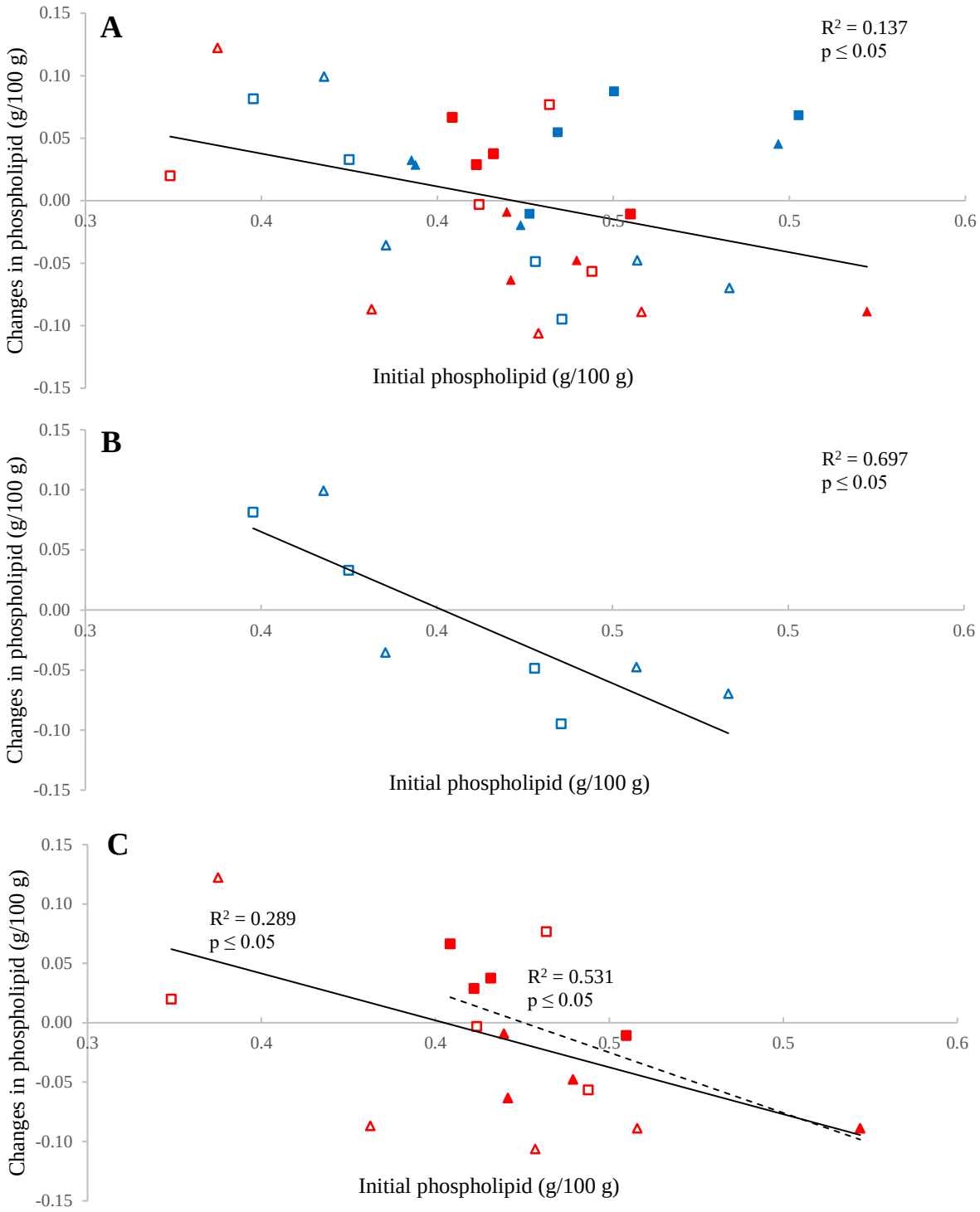

Figure 4.2 The relationship of initial phospholipid (PL) concentration and changes in PL concentration (vertical axis g/100 g) in post-smolt salmon white muscle after a four-week phase of starvation.
Prior to starvation, fish were fed two diets containing either fish oil (squares) or canola oil (triangles) as the main oil source until initial weights were doubled. Fish were held either at a control (15°C, CT) or elevated (20°C, ET) temperature, respectively. The anterior/dorsal cut (ADC, filled marker) and dorsal NQC (dNQC, open marker) fillet cuts were tested. This relationship was significant when all data was tested ($p < 0.05$) (A), in the dNQC of the CT treatments ($p < 0.05$) (B), in the ET treatments ($p < 0.05$, solid regression line) and for the ADC of the ET treatments ($p < 0.05$, scattered line), respectively (C).

may therefore indicate that these fish preferably used protein towards lipids at starvation. Further, elevated hepatic lipid catabolism was observed in several teleost species when acclimatised to high temperature (Johnston and Dunn, 1987). The decreased HSI in the ET treatments therefore indicate that these fish used larger amounts of lipid as energy substrate, which was in line with the decreased concentrations of total lipid and TAG in both fillet cuts in the ET treatments.

4.5.2 Effect of initial lipid composition on changes in lipids in white muscle during starvation

The significant relationship between initial total lipid concentration and changes in total lipid concentration in muscle during starvation at ET indicated that the amounts of total lipid used as an energy substrate increased with higher initial concentration. This is in line with previous suggestions that concentration of total lipid in muscle and temperature can affect the amounts of total lipid used during starvation (Einen et al., 1998). Further, this relationship was statistically stronger when the fillet cuts in the ET treatments were assessed separately. This indicated that there exist differences between fillet areas in their capacity to mobilise total lipid. The data points of the different dietary treatments thereby fitted well on the same regression line (Figure 4.1 B). This indicated that differences lipid composition in white muscle showed little impact on this relationship. The relationship of initial TAG concentration and changes in TAG concentration during starvation was almost identical to tota lipid, which showed that TAG was the main lipid source. This suggestion is reinforced by the significant ($p \leq 0.001$) relationship between concentrations of TAG and total lipid (Figure 4.4 A). However, the significant relationship of higher initial PL concentration and higher losses in PL concentration at starvation indicated that also PL was used as an energy substrate. This hypothesis was reinforced by the significant ($p \leq 0.001$) relationship of PL concentration and total total lipid concentration post starvation (Figure 4.4 B), which was not significant pre-starvation (Figure 3.4). The significant reduction in the proportions of 14:0, but also 16:1 FA and 18:1n9 in PL showed that these FA types were used for energy. This may indicate that these FA have no important structural functions in PL, and that excess amounts deposited this FA are used as an energy substrate during starvation. These findings were not affected by diet, indicating that there may be little potential to manipulate the type of FA used from PL as energy substrate during phases of starvation via dietary manipulation prior to starvation.

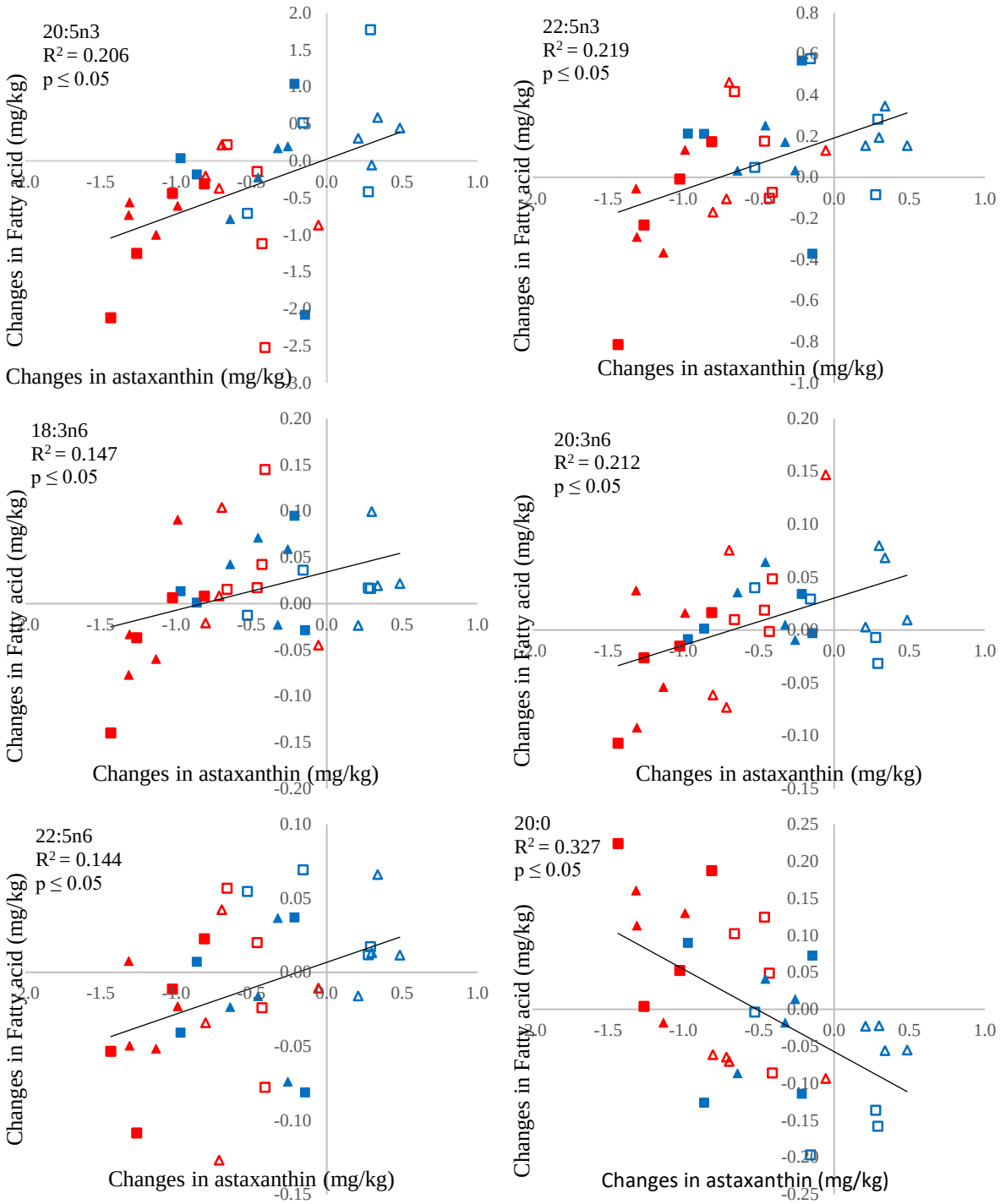

Figure 4.3 Relationship of changes in Ax concentration (horizontal axes, mg/kg muscle) and changes of individual fatty acids (FA) in triglycerides (TAG) (vertical axes, g FA/100g TAG) in post-smolt salmon white muscle after a four-week phase of starvation.
Prior to starvation, fish were fed two diets containing either fish oil (squares) or canola oil (triangles) as the main oil source until initial weights were doubled. During feeding and starvation, fish were held either at moderate (15°C, blue) or elevated (20°C, red) temperature, respectively. The anterior/dorsal fillet cut (filled marker) and dorsal NQC (open marker) were tested.

4.5.3 Patterns of astaxanthin loss

The reduction of Ax concentration in muscle of fish starved at ET was expected and the patterns of pigment loss along the fillet coincided well with patterns *in situ* after a period of high temperature and patterns (Grünenwald et al., *In preparation*). No changes in the pigmentation of Atlantic salmon occurred after starvation for up to 110 days at cold temperature (Einen et al., 1999; Einen and Thomassen, 1998). It was therefore rather surprising that loss of Ax concentration in the current study was also observed in the ADC at the CT. Possible explanations for this discrepancy are differences in the sample areas tested and much lower ambient temperatures (3.2 - 4.9°C) in the previous studies (Einen et al., 1999; Einen and Thomassen, 1998). Our findings showed that mechanisms leading to pigment depletion are facilitated by starvation and that ET exacerbated the effect.

4.5.4 Relationships between changes in astaxanthin and changes in lipids

Neither of the relationships between the changes in concentrations of total lipid, PL or TAG and changes in concentration of Ax was significant. Vegusdahl et al. (2004) examined the metabolism of ^{14}C-FA in cultured myotubes of Atlantic salmon and found that substrates of the long chain ^{14}C-FA tested were found esterified in nonpolar lipids and PL in the culture media. The myotubes also showed high mRNA expression of the most abundant protein-type in high density lipoprotein, which, serves as a blood carrier of lipids and Ax in salmon and is found long after feed intake ceased (Vegusdahl et al., 2004). During spawning migration, salmon cease feeding and reallocate muscle deposited FA and Ax into other tissues. The authors therefore hypothesised that the reallocation of excess lipids and Ax from muscle cells may be facilitated by the transport in high density lipoprotein formed in the muscle cells (Vegusdahl et al., 2004). However, due to the lacking relationship between loss of Ax and loss of TAG concentrations, we suggest that this mechanism did probably not explain the loss in Ax concentration in our study. Further, high density lipoprotein showed about three-fold higher affinity for 18:1n9 than for 22:5n3 in salmon (Torstensen et al., 2004). In the current study, there was a significant relationship between loss in the concentration of 22:5n3 in TAG and loss of Ax concentration in our study. Further, the concentration of 18:1n9 in TAG increased in the ADC at ET, where the highest loss in Ax concentration after starvation was observed. Together, these findings reinforced that loss of Ax in our study was probably not mediated by increased high density lipoprotein synthesis in muscle cells.

The changes in FA composition in TAG generally offered a surprising picture in the selection of FA as energy substrate. It was demonstrated in feeding salmon, that certain fatty acids, inclusive18:1n-9, 18:2n6, 18:3n3 are preferentially used as energy substrate (Nordgarden et al., 2003; Stubhaug et al., 2007). Further, essential FA like 20:5n3 and 22:6n3 are conserved for deposition, unless supplied in high dietary concentrations (Codabaccus et al., 2012; Stubhaug et al., 2007). The D1 contained higher concentrations of these FA. Nevertheless, proportions of 20:5n3 and 22:6n3 in TAG were significantly reduced in the ADC post-starvation regardless of dietary treatment, whereas the opposite was observed for proportions of 18:1n9 in TAG. The two major systems for β-oxidation are the mitochondrial- and the peroxosimal pathway and both pathways showed high FA selectivity (Frøyland et al., 1998). The FA 18:1n9 is oxidised in 44% higher rates than 22:6n3 in mitochondria (Henderson, 1996). We therefore suggest that our results may have indicated a downregulation of the mitochondrial pathway. In contrast to the current study, the concentration of n3-PUFA in muscle of Tasmanian Atlantic salmon post-smolt was increased after only 7 d starvation at 15°C (Codabaccus et al., 2012). This may indicate that the downregulation of the mitochondrial pathway may start first in advanced stages of starvation. In the current study the relationship between the loss in Ax concentration and the loss of the proportions (g FA/100 g TAG) of PUFA: 20:5n3, 22:5n3, 18:3n6, 20:3n6, 22:5n6 and sum n3 within TAG was significant. In rat livers, PUFA are more readily oxidised via the peroxosimal pathway

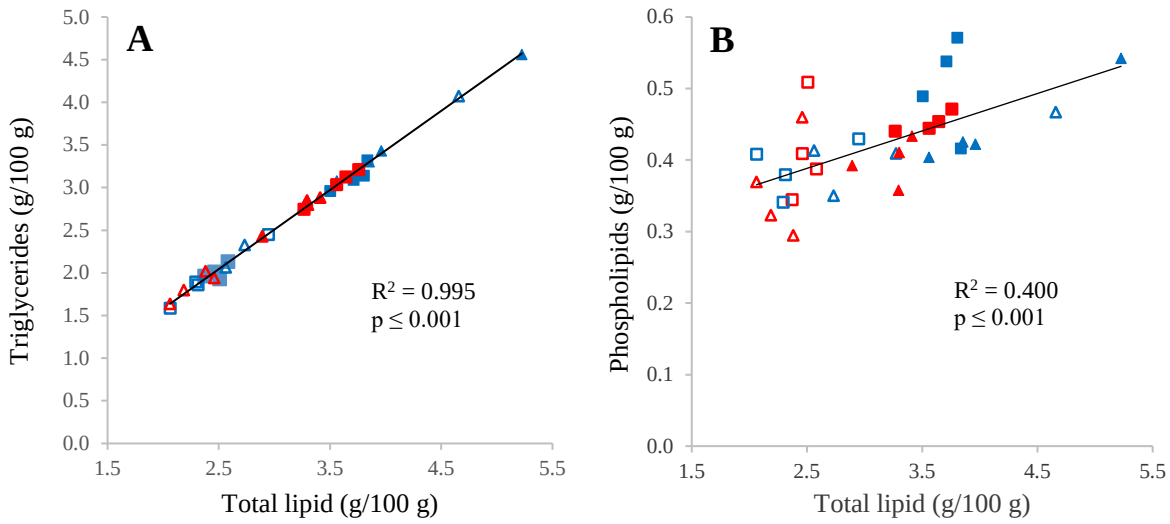

Figure 4.4 Relationship between total lipid concentration and concentrations of triglycerides (A) or phospholipids (B) in post-smolt salmon white muscle after a four-week phase of starvation.
Prior to starvation, fish were fed two diets containing either fish oil (squares) or canola oil (triangles) as the main oil source until initial weights were doubled. During feeding and starvation, fish were held either at moderate (15°C, blue) or elevated (20°C, red) temperature, respectively. The anterior/dorsal (filled marker) and dorsal NQC (open marker) were tested. Both relationships were highly significant.

compared to the mitochondrial pathway. Furthermore, the proportions of 22:5n3 and 22:6n3 in rainbow trout white muscle and liver were decreased in concurrence with increased activity of the peroxosimal pathway (Du et al., 2004). Together, our findings may indicate that increased loss of Ax was associated with the fortified activation of the peroxosimal, instead the mitochondrial pathway for β-oxidation.

Cellular lipid homeostasis can be highly complex and different systems involved in lipid catabolism are often interconnected (Christian et al., 2013; Singh et al., 2009). Autophagy is believed the major system that regulates energy homeostasis in starving mammals and can regulate lipid homeostasis via several mechanisms. These iclude the direct breakdown of lipid deposits (Christian et al., 2013; Singh et al., 2009), or indirectly via selective autophagy of mitochondria and peroxisomes (Rabinowitz and White, 2010). The latter mechanisms may offer a potential mechanism as to how the switch in lipid metabolism in the current study may have been facilitated. Autophagy is the process when cytosolic components are transported to and catabolised to lysosomes, which are rich in the cathepsins (Sandri, 2011). A previous study demonstrated the importance of autophagosomal/lysosomal system in muscle of starving rainbow trout (Seiliez et al., 2010) and a study with starving Atlantic salmon demonstrated temperature dependent expression of cathpesin L in muscle (Carter et al., 2008). It therefore appears that several systems that regulate energy homeostasis could be involved in pigment depletion in starving salmon. However, how these systems interact and how temperature and starvation interact to affect energy homeostasis in teleost fish is still poorly understood. We therefore warrant future studies with the aim to identify mechanisms that facilitate pigment depletion, to examine the effects of temperature and starvation on activity of different systems regulating energy homeostasis in white muscle.

4.6 Summary and Conclusion

We demonstrated that the concentration of total lipids, triglycerides and phospholipids in white muscle affected the use of these constituents during four-week starvation. This was more prominent at ET and the fillet areas tested showed differences in the amounts of total lipid and triglycerides used. Feeding diets with different oil blends prior to starvation showed only little impact on these features. The loss of Ax concentration was higher in the anterior/dorsal fillet cut and also higher at ET. The preferential use of fatty acid types as an energy substrate that are usually conserved in salmon could be an indication for a switch of lipid metabolism in white muscle tissue. The reduction of these fatty acids was associated

with the reduction in the concentration of Ax. Future studies with a focus on changes of different systems involved in energy metabolism in white muscle during starvation may help to identify processes that facilitate pigment depletion.

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CHAPTER 5

Levels of oxidative stress and loss of astaxanthin in different fillet areas of Atlantic salmon *Salmo salar* L. post-smolt starved at two temperatures

5.1 Abstract

Reduced pigmentation quality in Atlantic salmon fillet during phases of elevated temperature and the cessation of feed intake is a major commercial issue in Tasmania. The current study tested the reduction in the concentration of astaxanthin (Ax) in Atlantic salmon post-smolt muscle in two fillet cuts (anterior/dorsal cut, ADC and the dorsal Norwegian quality cut, dNQC) after four weeks starvation at an elevated temperature (20.8°C, ET) and a control temperature (15.3°C, CT), respectively. In order to determine the roles of oxidative stress (OS) and metabolic conversion of Ax, OS indices were analysed in muscle and liver, and idoxanthin (Ix) was analysed in muscle, respectively. Further, in order to test the effect of lipid composition in muscle on these parameters, fish were prior fed two diets containing either fish or canola oil as the major lipid source until they doubled initial weights at similar temperatures as starved (CT; 15.2°C and ET; 19.4°C). Fish were analysed before and after starvation. After starvation, the concentration of Ax decreased in both fillet cuts at ET, which was more pronounced on the ADC, and at CT, the Ax concentration was reduced exclusively in the ADC. Diet did not show an impact on OS or the reduction in the concentration of Ax and no Ix was detected. In contrast to other teleost species, the OS level was vastly decreased post-starvation in liver and muscle, and OS was higher in the ET treatments. Despite differences in Ax loss, there were no differences in OS between the fillet cuts within treatment. Further, the relationship between the reduction of Ax and OS in muscle at both temperatures was poor, indicating that OS did not cause the loss in Ax concentration. Marked differences in the life cycle of salmon compared with other teleost fish are proposed as an explanation for differences in the progression of OS during starvation.

5.2 Introduction

Pigmentation quality in salmon is enabled by the deposition of pigment carotenoids like astaxanthin (Ax) into muscle, which cannot be synthesised *de novo* and must therefore be supplied via diet. Salmon farmers around Tasmania experience reduced pigmentation quality around the summer period, which is characterised by reduced red colour intensity and increased heterogeneity in pigmentation on the fillet surface of individual salmon, leading to substantial product downgrade (T. Fox-Smith, pers. communication). Sea water temperatures around Tasmania commonly exceed 19°C for prolonged periods and reach over 23°C in some places (Stehfest et al., 2017). This phenomenon is concomitant with decreased feed intake or

even the cessation of feed intake (T. Fox-Smith, pers. communication), indicating that starvation represents an important factor in this phenomenon.

Carotenoids are potent antioxidants and previous studies have reported antioxidant activity of Ax in Atlantic salmon (Bell et al., 2000; Christiansen et al., 1995). Several studies found an increase in oxidative stress (OS) in livers of teleost fish during prolonged phases of starvation (Bayir et al., 2011; Morales et al., 2004; Pascual et al., 2003; Zhang et al., 2008; Zheng et al., 2016). This may indicate that the use of carotenoids at periods of increased OS during starvation may facilitate pigment depletion. OS is a result of a misbalance between the generation of so-called reactive oxygen species (ROS), which are a side product of aerobic metabolism and OS is the condition when the amount of ROS produced exceeds the amount eliminated (Lushchak, 2011). The liver shows high rates of metabolism and is generally a good indicator for the OS level in higher organisms (Furné et al., 2009). However, OS levels in white muscle and liver progressed differently in starving rainbow trout (*Oncorhynchus mykiss*) (Furné et al., 2009). Hence, the impact of starvation on OS can be tissue-specific and must be analysed directly in the tissue of interest. OS in white muscle is usually analysed in a defined sample area, or the whole fillet. Hence, little is known about differences in the OS level in white muscle of different fillet areas in fish. In Atlantic salmon, the white muscle is the proportionally largest tissue, and accounts for about 50 - 60% of the total body weight (Hamre and Lie, 1995; Page and Davies, 2006). In immature rainbow trout, white muscle from different fillet areas showed pronounced differences in muscle fibre density, levels of lipids and glycogen, and oxidative capacity (Kiessling et al., 1990; Kiessling et al., 1991). Further, significant differences in energy metabolism in white muscle from different fillet-locations were observed in starving sockeye salmon (*Oncorhynchus nerka*) during spawning migration (Kiessling et al., 2004). It is therefore conceivable that differences in the level of OS between different fillet areas exist. It was therefore hypothesised that differences in pigmentation between different fillet areas may be associated to differences in OS in starving salmon.

Long-term starvation is used as a tool to achieve production quota in Atlantic salmon production (Einen et al., 1998) and is therefore not exclusively associated with elevated temperature. However, temperature itself can considerably affect the OS level in ectotherms, since increasing temperature gives rise to higher metabolic activity, leading to increased production of ROS (Lushchak, 2011). Furthermore, the dietary oil type can affect the OS susceptibility in salmon muscle (Østbye et al., 2011). However, I am not aware about a study tested how dietary oil type, starvation and temperature interact to influence OS in fish.

Pigmentation quality in salmon can also be affected by the metabolic conversion of pigment carotenoids into colourless products. Previous studies reported that the amount of idoxanthin (Ix), the first metabolic product of Ax in salmonids, can be high and even exceed the concentration of Ax in muscle of salmon following the consumption of dietary Ax (Bjerkeng and Berge, 2000; Schiedt et al., 1989; Schiedt et al., 1988; Ytrestøyl et al., 2005). The conversion of Ax to Ix may be exacerbated by stress (Schiedt et al., 1989). However, it is not known if ambient temperature and/or starvation affect the metabolic conversion of Ax.

The current study tested how dietary lipid, temperature and starvation interact to influence the levels of OS and metabolic conversion of Ax to Ix in two fillet cuts in Atlantic salmon post-smolt. These features were compared with changes in the concentration of Ax during starvation to examine their involvement in pigment depletion.

5.3 Materials and methods

5.3.1 Fish husbandry, ethics, diets and experimental design

The experiment was conducted at the University of Tasmania's (UTAS) aquaculture research facility (IMAS Launceston, Tasmania). The UTAS Animal Ethics Committee in accordance with the "Australian code for the care and use of animals for the scientific purposes" approved the care and use of fish for this experiment (approval number A0014015). The average control temperature (CT) was 15.2°C (± 0.42 standard deviation, SD) and the average elevated temperature (ET) was 19.4°C (± 0.26 SD) after initial heat increment (see section 4.2.2) in the feeding phase. In the starvation phase, the average temperature in the ET treatments increased negligibly to 20.8ºC due to poorer performance of the heat control unit. Two diets were used to manipulate the lipid composition of white muscle in the preconditioning phase and contained either fish oil (D1) or canola oil (D2) as the main oil source (Table 5.1). The two oil blends were added onto batches of the same 4 mm kernel material (Skretting, Cambridge, TAS) by vacuum coating. The full sinking diets contained about 50 mg/kg Ax. There were four treatments: Fish oil at control temperature (D1CT), fish oil at elevated temperature (D1ET), canola oil at control temperature (D2CT) and Canola oil at elevated temperature (D2ET), respectively (n = 4 tanks/treatment).

5.3.2 Preconditioning phase

Atlantic salmon post-smolt (212 g ± 46 SD) were distributed into an experimental recirculating aquaculture system (RAS) (16 x 350L tanks) equipped with solids filtration, biological filtration, foam fractionation, UV disinfection and temperature control. Initial water temperature was 15.2°C and salt content 20‰ (salinity was incrementally increased to 24‰ within two weeks after start of the preconditioning phase). The temperature in the CT treatment tanks located in one system line (8 tanks) was kept at 15.2°C, and the temperature in one system line was incrementally elevated over seven days to 19.4°C (ET). Each of the two experimental diets were hand-fed to quadruplicate tanks at both temperatures in the morning and afternoon to apparent satiation. The ET treatments were fed three additional days in order to obtain similar final weights as the CT treatments at the end of the preconditioning phase, leading to 53 and 56 days of feeding for the CT and ET treatments, respectively.

Table 5.1 Ingredient-, oil blend-, and chemical composition and astaxanthin concentration in the experimental diets

Ingredients (g/100 g)		
Poultry meal		25.1
Fish meal		20.0
Feather meal		10.0
Wheat whole		5.0
Meat meal		9.2
Wheat gluten		3.8
Soy protein concentrate		2.5
Lupins dehulled		2.0
Blood meal		2.0
Minerals, vitamin and amino acid premix		1.2
Astaxanthin (10%)		0.1
Oil blend		19.1
	Diet 1	Diet 2
Fish oil		
% of oil blend	71.3	28.8
% of diet	13.6	5.5
Poultry oil		
% of oil blend	14.9	7.1
% of diet	2.8	1.4
Canola oil		
% of oil blend	13.8	64.1
% of diet	2.6	12.2
Chemical composition (g/100 g)		
Moisture	8.9	8.5
Crude protein	49.2	49.1
Total lipid	20.9	21.7
Ash	9.31	9.74
Astaxanthin (mg/kg)	56.4	47.1

5.3.3 Starvation phase

After the preconditioning phase, fish were starved for four weeks in their tanks at similar temperatures (see section 4.2.1) as the respective treatments were held at during the preconditioning phase.

5.3.4 Sampling

Fish were sampled at the end of the preconditioning phase (pre-starvation) and at the end of the starvation phase (post-starvation), respectively. All fish were thereby anaesthetised in their respective tanks, individually weighed and fork length measured, before six fish per tank were randomly taken and sacrificed for tissue collection. These fish were killed by an overdose, before the livers were excised, weighed and frozen at -80°C before analysed for glutathione (GSH) and glutathione disulphide (GSSG). The right hand side fillet was taken, boned, skinned and cut along the horizontal septum. The dorsal fillet half was then cut longitudinally into six equally long cuts and the first (anterior dorsal cut; ADC) and fourth (dorsal part of the NQC; dNQC) fillet-cuts from anterior to dorsal from each tank were pooled for fillet cut and homogenised with a food processor for 11 sec. Then, subsamples were frozen in liquid nitrogen and stored at -80°C until analysed for Ax, Ix classes and FA within TAG and PL. Another subsample of the pooled fillet cut-homogenates was frozen at -20°C until analysed for chemical composition.

5.3.5 Chemical analysis

All analysis were conducted in duplicate.

Astaxanthin and idoxanthin in white muscle

Pre-homogenised muscle (1 g) was mixed with 4 mL of acetone, cooled to -20°C and homogenised using Precellys standard tubes containing 2.8 mm ceramic beads (lysing kit CK28, Bertin Technologies, France), before the mix was centrifuged at 3,000 g for 5 min. For the analysis of idoxanthin, 2 μL of sample was injected into a reverse phase HPLC system (model 1200, Agilent) using a mix of water/methanol/acetonitrile (18/20/62, v/v/v) as fluid phase and a Halo C8 column (2.7 μm, 150 x 4.6 mm, Advanced Materials Technology). The flow rate was 1.5 mL/min. The instrument setup was calibrated with an all-E idoxanthin

standard (DSM Nutritional Products) and it was ensured that Ix was well separated from Ax on the chromatograms. For the analysis of astaxanthin, 2 mL of the acteone extract was evaporated under N_2-gas at 50°C, and the vials were then cooled to room temperature before the samples were dissolved in 2 mL of a mixture of n-hexane/acetone (86:14, v/v). Then, 100 uL of this extract was injected into a normal-phase HPLC via by normal Phase HPLC (model 1200, Agilent) and subsequent UV/VIS-detection at 470 nm wavelength. Analytical conditions and calibration for Ax were conducted as described previously (Schüep and Schierle, 1995), and all-E Ax standards were used for calibration (LGC, Germany).

Astaxanthin in diets

Astaxanthin in diets was analysed by normal phase HPLC (model 1200, Agilent) and subsequent UV/VIS-detection at 470 nm wavelength. The instruments were calibrated using all-E standards (LGC Standards GmgH, Wesel, Germany) and standard concentrations were confirmed by spectrophotometry (Cary 50, Varian). All procedures were previously described in detail (Schüep and Schierle, 1995).

Glutathione and glutathione disulphide in livers

About 200 mg of each of the livers taken from the same tank at a given sample time were transferred in a vial containing 4 mL of ice cold 1.15% (w/w) KCl solution, then homogenised for 15 s (IKA Ultra Turrax T10, basic). After homogenisation, the remaining extraction steps were conducted as described previously (Bouligand, Deroussent, Paci, Morizet & Vassal, 2006), using 50 µL of a stable isotope standard solution containing GSH (Glycine-13C2, 98%+; 15N, 96-99%; 65-70% Net; conc.: 60 µg/mL) and GSSG (Glycines-13C2, 98%+; 15N, 96-99%; 65-70% Net; conc.: 130 µg/mL, both standards were purchased from Novachem Pty Ltd, Collingwood, VIC, Australia). Extracts were stored at -80°C until analysed. The thiol GSH (and surrogate standard) were analysed as their carboxymethyl (CM) derivatives, while GSSG (and surrogate standard) were analysed as free compounds. Analyses were conducted using a Waters Acquity H-Class UPLC instrument coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was used, held at 35°C. The mobile phase consisted of 0.1 % (v/v) formic acid (solvent A) and acetonitrile (solvent B). Initial conditions were 100% A, decreasing to 20% A by 3.5 min, which was maintained for 0.5 min before returning to 100% A over 0.5 min and re-equilibration for 3 min. The flow rate was 0.35 mL/min. The injection

volume was 10 µL. The MS/MS was operated in positive ion electrospray mode, with capillary voltage of 2.7 KV, and individual cone voltages / collision energies for each multiple reaction monitoring (MRM) transition. The desolvation temperature was 450°C.

Malondialdehyde in white muscle

Pooled and pre-homogenised muscle tissue (about 250 mg) was transferred into a glass vial on ice containing 2 mL of 1% (v/v) sulphuric acid conc. in PBS, before 20 µL of a deuterated stable isotope surrogate standard (MDA-D2) solution was added (concentration 5.0 µg/mL). The MDA-D2 surrogate standard was derived from hydrolysis of 1,1,3,3-tetraethoxypropane; 1, 3-D2 (purity: 95%; Novachem, Collingwood, VIC, Australia) with 1% (v/v) sulphuric acid at room temperature in the dark for 2 h. This mixture was subsequently homogenised for 30 s (IKA Ultra-Turrax T10 basic), before 1 mL of homogenate-solution was transferred into a snap-cap vial and centrifuged at 10000 rpm for 30 min at 4°C. The remaining extraction steps were conducted as described previously (Faizan, Esatbeyoglu, Bayram & Rimbach, 2014), using a 1.2 mM dinitrophenylhydrazine (DNPH) in acetonitrile/formic acid (98/2, v/v) for MDA and surrogate standard derivatisation prepared fresh daily. Extracts were stored at -80°C until analysed. MDA and surrogate standard (MDA-D2) were analysed as their DNPH derivatives. UPLC-MS/MS conditions were identical to the GSH analysis method (see above), apart from the following. The mobile phase consisted of 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). Initial conditions were 60% A, held for 1 min before a gradient to 20% A by 3 min, held for 1 min, prior to returning to initial conditions and re-equilibration for 3 min.

Chemical composition of diets

Moisture content was determined gravimetrically by freeze drying to constant weight, total lipid was analysed according to Bligh and Dyer (1959) and ash was determined by sample combustion at 600°C for 6 h. The Kjehldahl method was used to measure nitrogen (N), using sulphuric acid (>98 %) and a copper catalyst for digestion (400°C for 2 h). Crude protein (CP) was calculated as = N × 6.25. All samples were analysed in duplicate.

5.3.6 Calculations and statistical analysis

All results presented in tables were compared using 3-way ANOVA to test the effects of the factors sample time, temperature and diet, and Tukey HSD was used to determine treatment differences. The difference in the concentrations of MDA between the fillet cuts of a treatment within sample time was tested by one way ANOVA. All statistical analyses in tables was based on treatment means (± SEM), and % change-data is displayed, but not included in the analyses. Homogeneity of variance was evaluated by examination of residual plots and data was $\log_{10}$-transformed, if necessary. The relationship between loss in Ax concentration during starvation and MDA concentration post-starvation in muscle was tested by linear regression analysis. Statistics software used was SPSS version 24.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). The level of significance was set at $p \leq 0.05$.

5.4 Results

The Ax data set presented in Table 5.2 was reproduced from chapter 4. The Ax concentration

Table 5.2 Changes in the concentration of astaxanthin (Ax, mg/kg), and malondialdehyde (µmol/g) in white muscle from two fillet cuts (anterior/dorsal cut, ADC; and dorsal NQC, dNQC) of post-smolt salmon before (Pre) and after (Post) a four-week phase of starvation.
Prior to starving, fish were fed diets either containing fish oil (D1) or canola oil (D2) as the main lipid source until initial weights were doubled. Fish were held either at a control (15°C, CT) or elevated (20°C, ET) temperature, respectively.

	A	Ax ADC	Ax dNQC	MDA ADC	MDA dNQC
Pre	D1CT	^{ab}1.81 ± 0.08	^{bc}1.73 ± 0.23	^{abc}24.4 ± 1.5	^{ab}28.2 ± 1.8
	D2CT	^{ab}1.77 ± 0.09	^{abc}1.77 ± 0.11	^{ab}29.2 ± 2.3	^{abc}26.7 ± 0.5
	D1ET	^{ab}1.78 ± 0.12	^{ab}2.39 ± 0.12	^{a}31.3 ± 1.7	^{abc}27.9 ± 2.0
	D2ET	^{a}1.89 ± 0.12	^{a}2.44 ± 0.15	^{ab}27.6 ± 2.0	^{a}30.6 ± 2.5
Post	D1CT	^{c}1.26 ± 0.14	^{c}1.70 ± 0.10	^{c}18.2 ± 1.4	^{e}17.0 ± 0.1
	D2CT	^{bc}1.35 ± 0.09	^{abc}2.10 ± 0.12	^{c}16.6 ± 2.5	^{de}18.3 ± 0.8
	D1ET	^{d}0.65 ± 0.05	^{abc}1.90 ± 0.12	^{bc}21.2 ± 1.5	^{cde}21.4 ± 0.8
	D2ET	^{d}0.70 ± 0.10	^{abc}1.87 ± 0.14	^{bc}21.6 ± 1.1	^{bcd}22.7 ± 1.8
%Change	D1CT	-30.4	-1.7	-25.2	-39.8
	D2CT	-23.7	18.7	-43.2	-31.4
	D1ET	-63.7	-20.5	-32.2	-23.2
	D2ET	-62.9	-26.0	-22.0	-26.0
[2]*3-Way ANOVA*					
Sample time (S)		***	N.s.	***	***
Temperature (T)		***	**	*	**
Diet (D)		N.s.	N.s.	N.s.	N.s.
Interactions		S × T***	S × T**	S × D × T*	N.s.

Values presented as treatment mean (n = 4) ± SEM. Values in the same column with different superscript letters are significantly different (Tukey HSD, $p \leq 0.05$). [1]% change during starvation
[2]N.s.: Not significant *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

in muscle decreased in the ADC post starvation ($p \leq 0.001$) and was lower at ET post-starvation, respectively ($p \leq 0.001$). In the dNQC, the concentration of Ax was higher in the ET group pre-starvation ($p \leq 0.005$). The concentrations of all OS indices in muscle and liver were significantly decreased post starvation ($p \leq 0.001$) (Tables 5.2, 5.3). Further, apart from hepatic OSI, all OS indices were overall increased in the ET group and all hepatic OS indices were significantly increased in the ET group post starvation. No significant differences in MDA concentration were found between the fillet cuts tested and no Ix was detected in any of the samples.

5.5 Discussion

This Chapter presents the OS parameter of the same fish tested in Chapter 4. No significant effects of the factor diet and thus, dietary oil type were observed on the parameters tested, and the relationships of dietary lipid, and local lipid composition in white muscle on OS was discussed in detail in Chapter 3. Further, no Ix was detected in the samples and the possible

Table 5.3 Levels of glutathione (GSH), glutathione disulphide (GSSG) and total GSH (µmol/g) and oxidative stress index (OSI) in livers of post-smolt salmon before (Pre) and after (Post) a four-week phase of starvation. Prior to starving, fish were fed diets either containing fish oil (D1) canola oil (D2) or as the main lipid source until initial weights were doubled. Fish were held either at a control (15°C, CT) or elevated (20°C, ET) temperature, respectively.

		GSH	GSSG	Total GSH[1]	OSI[4]
Pre	D1CT	^{ab}2.16 ± 0.18	^{a}2.85 ± 0.40	^{a}7.85 ± 0.98	^{a}72.1 ± 1.2
	D2CT	^{ab}2.11 ± 0.08	^{a}2.73 ± 0.19	^{a}7.57 ± 0.42	^{a}72.0 ± 1.1
	D1ET	^{ab}2.13 ± 0.08	^{ab}2.61 ± 0.19	^{a}7.36 ± 0.43	^{a}70.9 ± 1.3
	D2ET	^{a}2.29 ± 0.06	^{a}2.78 ± 0.21	^{a}7.85 ± 0.35	^{a}70.6 ± 2.2
Post	D1CT	^{c}1.33 ± 0.04	^{c}1.07 ± 0.07	^{c}3.48 ± 0.10	^{b}61.5 ± 2.1
	D2CT	^{c}1.30 ± 0.10	^{c}1.03 ± 0.04	^{c}3.36 ± 0.18	^{b}61.5 ± 1.4
	D1ET	^{ab}1.97 ± 0.06	^{abc}2.01 ± 0.21	^{ab}5.99 ± 0.47	^{ab}66.9 ± 1.5
	D2ET	^{b}1.83 ± 0.08	^{bc}1.68 ± 0.17	^{b}5.19 ± 0.32	^{ab}64.3 ± 2.8
% Change	D1CT	-38.4	-62.3	-55.8	-14.6
	D2CT	-38.5	-62.2	-55.6	-14.5
	D1ET	-7.7	-23.0	-18.5	-5.7
	D2ET	-20.0	-39.6	-33.9	-8.9
[3]3 Way-ANOVA					
Sampling time (S)		***	***	***	***
Temp (T)		***	*	***	N.s.
Diet (D)		N.s.	N.s.	N.s.	N.s.
Interactions		S × T***	S × T*	S × T***	S × T*

[1]GSH + 2 GSSG
[2](2 GSSG / (GSH + 2 GSSG)) × 100
[3]N.s.:Not significant, *$p \leq 0.05$, ***$p \leq 0.001$

effect of genetic isolation of Tasmanian salmon on the conversion from Ax to Ix was also discussed in Chapter 3. The discussion of the current Chapter therefore exclusively addresses OS and the effects of OS on the reduction of Ax concentration.

5.5.1 The effect of oxidative stress on pigment depletion

In contrast to a previous study (Furné et al., 2009), but in line with another (Nakano et al., 2014), the patterns of OS indices measured in muscle and liver showed similar responses within all treatments, even though the interactions between temperature and sample time were only significant in the hepatic OS indices. The overall increased level of OS in the ET group confirmed that the OS level in ectotherms usually increases with increasing ambient temperature (Lushchak, 2011; Nakano et al., 2014). Interconnections between Ax and the antioxidant α-tocopherol (A-toc), were demonstrated *in vitro* by antioxidant synergism and interactive sparing (Bell et al., 2000). A-toc is the main antioxidant against lipid peroxidation in fish (Hamre, 2011) and it was demonstrated that the dietary inclusion of Ax-rich yeast vastly reduced the concentration of lipid peroxides in muscle of trout fed a diet with oxidised lipids (Nakano et al., 1999). In our study, significantly higher concentrations of the lipid peroxidation product MDA were found in the ET group post starvation, which also showed higher losses in Ax concentration during starvation. One may interpret this as an indication for the involvement OS and pigment depletion. However, there was significant drop of MDA post-starvation and despite the differences in pigment loss between the fillet cuts, no significant differences between the fillet cuts in MDA were observed. Furthermore, the relationship between the loss of Ax during starvation and the concentration of MDA post starvation was poor (Figure 5.1). Together, these results indicated that the connection of higher Ax loss in coherence with higher concentrations of MDA post-starvation in the ET group was incidental, rather than causal.

5.5.2. Differences among salmon and other teleost fish, implication of differences in life cycle

The majority of studies that tested the effect of long-term starvation on OS in other teleost species than salmon reported increased OS at starvation (Furné et al., 2009; Morales et al., 2004; Pascual et al., 2003; Zhang et al., 2008; Zheng et al., 2016). The drop in OS in our study (see also chapter 2) was therefore surprising. Many teleost species are exposed to phases of starvation during their life history, e.g. due to seasonal fluctuations in food availability (Furné et al., 2009). However, the life cycle of anadromous salmonids

significantly differs from most other teleost species in the reproductive phase. The energy demand for salmonids at this life stage is immense due to: the adaption to intense fluctuations in salinity, temperature and currents in combination with formidable distances that must be travelled to the spawning sites, which is concurrent with voluntary fasting (Doucett et al., 1999). Fluctuations in salinity and temperature can lead to oxidative stress and increased muscular activity and exercise can also increase the OS level as previously reviewed (Lushchak, 2011; Monaghan et al., 2009). OS can reduce the reproductive potential (Monaghan et al., 2009). Hence, the reduction of OS during spawning migration may be a

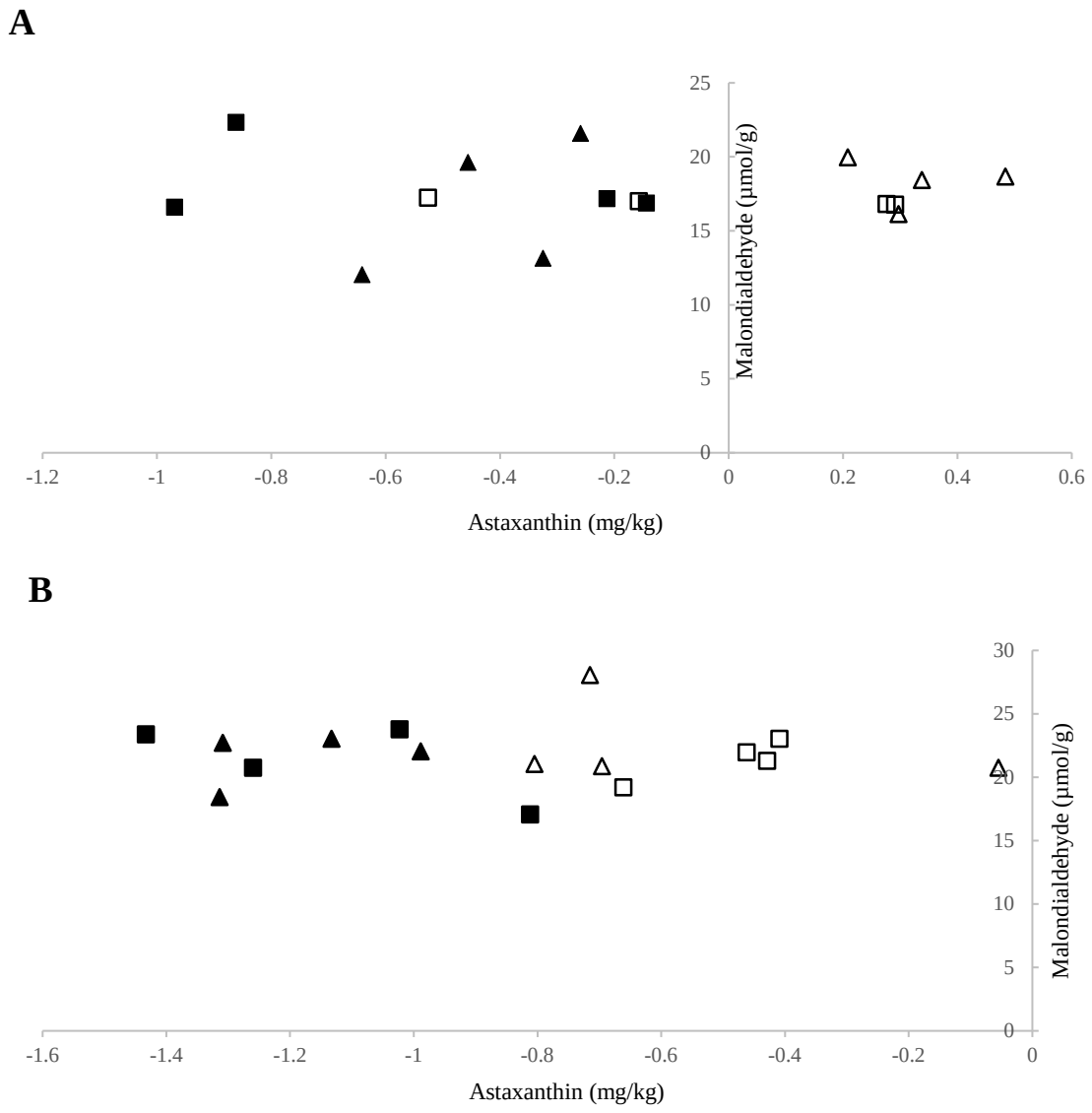

Figure 5.1 Relationship between loss of astaxanthin concentration (horizontal axes, mg/kg) and the concentration of malondialdehyde in white muscle of post-smolt salmon after a four-week phase at starvation. Prior to starvation, fish were fed two diets containing either fish oil (squares) or canola oil (triangles) as the main oil source until initial weights were doubled. Fish were held either at control (15°C, A) or elevated (20°C, B) temperature, respectively. The anterior/dorsal fillet cut (solid marker) and dorsal NQC (open marker) were tested. There was no significant relationship in either of the temperature groups ($R^2 = 0.001$, $p > 0.05$)

particularly important trait for the reproduction success in salmon. Our results may therefore be an indication for the development of advanced OS-defence mechanisms in salmon during starvation. Yet, due to the pronounced impact of feed intake on OS observed in our study, it is tempting to suggest that starvation itself may represent an evolutionary adaption of anadromous salmonids for the reduction of OS during spawning migration.

5.5.3 Importance of pigment carotenoid availability for reproduction success

It appears likely that if anadromous salmonids primarily relied on the exogenous antioxidants (those which cannot be synthesised *de novo*), instead of endogenous antioxidants during spawning migration, a depletion of exogenous antioxidants could occur quickly. Carotenoids play an important role in sex signalling during spawning. When utilised for ornamentation, carotenoids cannot be utilised for their use as antioxidants and even have to be protected from oxidation (Monaghan et al., 2009). Further, carotenoids are allocated to eggs and higher carotenoid levels in salmon eggs may have positive effects on survival and disease resistance in later life stages (Tyndale et al., 2008). The use and depletion of carotenoids for functions other than ornamentation and egg-allocation in the stage of reproduction would therefore directly have negative consequences for spawning success, offspring survivability and hence, the propagation of genetic material. This makes it seem unlikely that carotenoids in salmon are used as antioxidants to a higher extent at starvation, especially when taking into account the multitude of available endogenously produced antioxidants that are available (Lushchak, 2011).

5.6 Summary and conclusion

Our results indicated that the depletion of Ax in white muscle from different fillet areas of salmon starved at two temperatures was not associated with oxidative stress or metabolic conversion of Ax to Ix. The level of oxidative stress decreased at starvation, which is in contrast to other teleost species. In salmon, starvation is a natural feature during spawning migration. The development of oxidative stress levels at this life stage would jeopardise the reproduction success. Hence, reduced oxidative stress during starvation may be a result of evolutionary adaption. Further, the use of carotenoids as antioxidants would disable their use for other functions essential for reproduction success and offspring survival.

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CHAPTER 6

General Discussion

6.1 Overview

This thesis aimed to increase the knowledge about reduced pigmentation quality in salmon around the summer period that leads to a substantial commercial downgrade of Tasmanian salmon products. This is increasingly problematic in other salmon producing countries due increasing sea temperatures in recent years. Published literature to date offers very limited information about the deposition of pigment carotenoids into muscle at elevated temperature (ET). The same applies to the metabolic fate of muscle deposited pigment carotenoids in starving salmon. Hence, the research strategy adopted in the thesis was to examine broader objectives with the aim to improve the fundamental understanding of factors and mechanisms that facilitate reduced pigmentation quality in salmon exposed to ET and to improve the pigmentation quality in farmed salmon. This final chapter discusses the findings of the research in chronological succession of the two major phases that occur around the summer period on farms exposed to ET in summer: pigment deposition at ET when feed intake still occurs (Section 6.2), and pigment depletion in starving fish (Section 6.3). Subsequently, based on the findings from the research chapters, possible strategies for the improvement of pigmentation in summer are discussed (Section 6.4), and avenues that could be explored to deepen these findings are suggested (Section 6.5).

6.2 Pigment deposition in salmon feeding at elevated temperature

In Chapter 3, the concentrations of Ax in muscle were higher when salmon doubled their initial weight at ET compared to the control temperature. This finding was important, because it indicated that the reduced pigmentation quality in salmon exposed to ET is likely not due to the deteriorated pigment deposition when salmon are still feeding. However, differences in Ax concentration between the anterior/dorsal cut (ADC) and dorsal part of the NQC (dNQC) showed that differential pigment deposition along the fillet may contribute to the heterogeneous pigmentation at ET. The effect of temperature on pigmentation concentration in salmon muscle was previously suggested (Refsgaard et al., 1998), to the best of my knowledge, the results presented in Chapter 3 provide the first experimental demonstration that temperatures can significantly affect pigment concentrations in different fillet cuts in salmon. In fact, the difference in carotenoid concentration between the fillet cuts at ET (Chapter 3) may indicate that differences in temperatures between studies testing salmon in the marine environment under commercial conditions, may explain why differences in carotenoid concentrations between longitudinal fillets cuts were observed in some studies

(Refsgaard et al., 1998; Skjervold et al., 2001), but not in another study (Bell et al., 1998). Further, these novel findings emphasise the importance in the choice of fillet sample cut for future studies testing the pigmentation in salmon at ET. Nevertheless, due to differences in pigment deposition in salmon of different sizes (Section 1.4.2), our results still need to be confirmed in harvest sized fish.

The differences in Ax deposition between the fillet cuts and temperature groups were not associated with oxidative stress (OS), metabolic conversion of Ax to Ix, or dietary oil blend (Chapter 3). However, the differences in the concentration of Ax per unit of crude protein observed between the two fillet cuts at ET could be an indication for the involvement of changes in the affinity of myofibrillar proteins to bind carotenoids. Comparison of muscle fibre density and colour score in smoked salmon unveiled a significant correlation between these parameters (Johnston et al., 2000). However, the relationship of muscle fibre density and colour visualisation (SalmoFan^{TM}- score per unit of pigment carotenoid in muscle) between wild and farmed Atlantic salmon was different, even though there was no difference in muscle fibre density between the two strains (Johnston et al., 2006). Hence, the relationship between colour visualisation and muscle fibre density is likely indirect and differences in the organisation of carotenoids in muscle proteins, caused by differences in pH and activities of proteolytic enzymes, could be an explanation (Johnston et al., 2006). Nevertheless, there are other mechanisms as to how processes in muscle proteins may affect pigmentation quality.

Pigment carotenoids, once deposited into muscle of immature fish, are considered to be relatively stable and not exposed to metabolism (Torrissen et al., 1995). Poor Ax deposition was observed in coho salmon (*Oncorhynchus kisutch*) when treated with triiodothyronine (T_3), which was due to higher protein turnover, and thus, opportunity for circulating Ax to be metabolised (March et al., 1990). An increase in ambient temperature could potentially initiate the same mechanisms (March et al., 1990). Dietary supplementation of T_3 mediated the accelerated transition of a myofibrillar protein in muscle of Arctic charr (Martinez et al., 1995). More interestingly, higher ambient temperature had a stronger impact on this feature than dietary T_3-supplementation (Martinez et al., 1995). There is a high inter-individual variability in pigmentation quality of salmon and the reasons for this phenomenon are poorly understood (Section 1.4.4). Another factor that shows high inter-individual variability in salmonids and other teleosts is protein turnover (Carter et al., 1993a; Carter et al., 1993b; McCarthy et al., 1994). Hence, difference in protein turnover or proteolytic activity between individuals may explain the phenomenon of the high inter-individual variability in

pigmentation success. To the best of my knowledge, differences in protein turnover in white muscle from different fillet areas of teleosts were not measured. However, significant differences in muscle fibre density, fibre development, and fibre hypertrophy exist between white muscle from different fillet areas in rainbow trout (Kiessling et al., 1990; Kiessling et al., 1991) and Sockeye salmon (*Oncorhynchus nerka*) (Kiessling et al., 2004). This makes it likely that also differences in proteolytic activity or protein turnover between white muscle from different fillet areas exist. It therefore seems conceivable that the significant differences in Ax concentration per unit crude protein between the fillet areas tested at ET (Chapter 3) were caused by differences in proteolytic activity or protein turnover, which led to differences in the affinity of white muscle proteins to bind carotenoids. This suggests that future studies should aim to elucidate factors that lead to heterogeneous pigment deposition along the fillet of salmon growing at ET, to examine the relationships between protein turnover and pigment deposition in white muscle of different fillet areas.

6.3 Pigment depletion in salmon starved at different temperatures

Arguably, the most important outcome of the current research was the identification of starvation as the key factor that triggered pigment depletion. There was an increase in the concentration of α-tocopherol and a reduction in the concentration of malondialdehyde in white muscle in concurrence with deceasing levels of hepatic OS following a heat/starvation-challenge (Chapter 2). The exact mechanisms as to how carotenoids function during episodes of OS and the metabolic product of carotenoids after their use in the prevention of OS in salmon remain to be determined. One might therefore suggest that the selection of OS indices in muscle examined in this thesis may not have been suitable or to imprecise to examine the involvement of OS in the deterioration of pigment carotenoids. Nevertheless, interactions between α-tocopherol and Ax, and prevention of lipid peroxidation after dietary carotenoid supplementation in salmonids were demonstrated in previous studies (discussed in the Chapters 2 and 5). This circumstance, but also the tight interconnections between carotenoids and other substances of the antioxidant network in salmon (Section 1.8), indicate that the indices chosen were suitable. Furthermore, considerable differences between Ax and Cx in their function as *in vitro* antioxidants were demonstrated (Miki, 1991; Shimidzu et al., 1996). Even though relationships between reactive oxygen species and the elimination of reactive oxygen species ise likely more complex *in vivo* (Monaghan et al., 2009), the fact that the loss in a*-value in fillets was similar between salmon loaded with either Ax, Cx or a mix (Chapter

2) may be an indication that OS did not cause the carotenoid depletion observed. The localised pigment depletion could have indicated that OS occurred only in these fillet areas and was overlooked, since white muscle from the whole fillet, instead of the areas affected by pigment depletion was analysed for the OS indices (Chapter 2). However, the non-significant relationship between the changes in concentrations of Ax during starvation and the concentrations of malondialdehyde post-starvation in the ADC and dNQC (Chapter 5) showed that this was likely not the case.

There was no Ix in white muscle of starving salmon, indicating that the conversion of Ax to Ix was not associated to the depletion of Ax (Chapter 5). One might argue that this result may differ for larger salmon, due to the pronounced effect of fish size on metabolic conversion of pigment carotenoids. Indeed, no Ix was detected in white muscle samples analysed from larger starved fish (1-3 kg) taken from a farm-based trial and a recirculating aquaculture system experiment (unpublished results). Ix is the first metabolite of Ax in salmon and can significantly contribute to the total carotenoid content (Chapter 5). However, pigment carotenoids in salmon were converted into a range of succeeding metabolites (Schiedt et al., 1988). After application of an oral dose of radiolabelled Ax, some of the radioactivity in salmon could not be explained by the presence of Ax and Ix, and was therefore due to other Ax-catabolites (Aas et al., 1999). Therefore, there is a chance that different catabolic pathways, or an accelerated conversion from Ix into the next metabolite, was involved in the pigment depletion in the current research. Further, analysis of pigment-carotenoids and metabolic products in organs that are important for carotenoid catabolism (especially liver, Section 1.4.2) in the current research would likely have enabled a better understanding of catabolic pathways of pigment carotenoids under the conditions tested. Hence, for future studies with the aim to elucidate these aspects, we recommend the use of mass balance approaches, ideally in combination with labelled carotenoids in order to enable analysis-coverage of all metabolites.

The manipulation of lipid composition in muscle did not affect pigment depletion in a subsequent starvation phase (Chapter 4). However, the differences in fatty acids (FAs) used as energy substrate indicated a switch from mitochondrial to peroxosimal β-oxidation in fillet areas with higher loss of Ax concentration. The circumstance that the loss of Ax in this experiment was significantly higher at ET indicated that ET exacerbated the effect, which may be due to higher energy demands at higher temperatures (Lushchak, 2011). The overall increased levels of OS indices at ET during starvation (Chapter 5) may reinforce this hypothesis, since the production of reactive oxygen species is linked to metabolic activity

(Section 1.9). It was more surprising that this switch occurred preferentially in the ADC. White muscle tissue serves as a critical reserve in starving animals and the prioritisation of muscle groups important for locomotion often dictate the muscle groups affected (McCue, 2010). White muscle tissue of rainbow trout taken from different vertical fillet areas showed distinctive differences in muscle fibre density, lipid and glycogen levels and oxidative capacity, indicating different functionalities (Kiessling et al., 1990). The switch in lipid metabolism between the fillet areas (Chapter 4) may therefore indicate functional differences in white muscle from different fillet areas, in their conservation for locomotion and use for energy during starvation. Hence, exacerbated pigment depletion may be found in white muscle from fillet areas primarily used for energy during starvation.

6.4 Potential strategies to improve pigmentation of salmon at elevated temperature

6.4.1 Maintenance of feed intake during phases of elevated temperature

The identification of starvation as the key factor leading to pigment depletion indicates that the manipulation of feed intake during the summer period may present a promising approach to prevent pigment depletion. Under non-limiting environmental conditions, salmon farmers keep feed rates as high as possible to support growth, but also to maintain high pigment carotenoid deposition in fillet, which is positively correlated to feed intake rates and condition (Ytrestøyl et al., 2004). The following paragraph proposes approaches that may procure the maintenance of feed intake in salmon at ET with the aim to prevent starvation-induced pigment depletion. One important question in this context is whether lower feed intake may be sufficient, or if high rates must be maintained during summer to prevent pigment depletion.

Reduced feed intake in salmon at ET was concurrent with alterations in the plasma concentrations of leptin and ghrelin, indicating that these hormones are of high importance in appetite regulation (Hevrøy et al., 2012; Kullgren et al., 2013). These are two of the key hormones for energy homeostasis in mammals (Meier and Gressner, 2004). The concentration of ghrelin was changed at starvation and affected by dietary total lipid concentration and showed positive correlation to liver and muscle total lipid concentration and condition factor in rainbow trout (Jönsson et al., 2007). In Atlantic salmon parr, restricted feeding changed the gene expression of leptin and leptin receptors genes and plasma leptin

concentration, showing that this hormone is involved in energy homeostasis in salmon as well (Trombley et al., 2012).

Restricted feeding of Atlantic salmon led to reduced endogenous storage of total lipid and to compensatory growth when satiation-feeding was restored, which may indicate lipostatic appetite regulation in salmon (Johansen et al., 2001). Endogenous lipid storage in salmon can be manipulated via dietary total lipid concentration or feed ration (Einen and Roem, 1997; Hillestad et al., 1998; Refstie et al., 2001). Compensatory growth and hyperphagia was also initiated in immature salmonids following periods of limited food supply, even for short periods (Nicieza and Metcalfe, 1997; Nikki et al., 2004). This may indicate that the reduction of dietary total lipid concentration, or restricted feeding prior to the peak of summer may present a potential tool to maintain appetence during the peak of summer or could delay the loss of appetence as temperatures rise. Whether this approach has merit will depend on whether the potency of mechanisms that drive compensatory growth can override the potency of mechanisms that cause reduced feed intake at ET. Reduced aerobic scope may limit the metabolic capacity to metabolise nutrients, and starvation may represent a coping-strategy at ET (Section 1.5). If these hypotheses hold true, there may be limited potential to influence feed intake at ET via the manipulation of nutritional status or body condition. In addition, the use of restricted feeding is associated with overall increased production costs due to suppressed growth and lower fillet yields (Einen et al., 1999). Further, reduced dietary total lipid concentration could lead to increased use of dietary crude protein for energy (Einen and Roem, 1997).

Senegalese sole (*Solea senegalensis*) brood stock at ET preferred diets rich in arachidonic acid, due to increased metabolic demand (Norambuena et al., 2012). Perhaps, the identification and dietary manipulation of functional ingredients for salmon at ET may be an alternative approach for the maintenance of feed intake in summer without restricted feeding or reduction of dietary total lipid concentration.

Another possible strategy to maintain feed intake at ET may be to identify fish families showing better appetence at ET as a selective breeding trait. The heritability of many traits that lead to a better adaption to ET in salmon appear to be high (Section 1.6). A recent study examined temperature-specific differences in growth-performance between commercial salmon strains, wild salmon populations originating from rivers with different seasonal temperatures, and hybrids of these strains (Harvey et al., 2016). The results indicated a potential for temperature adaption by the means of cross-breeding. Farm observations from routine samplings at ET in Tasmania suggested pronounced phenotypical inter-individual

differences in performance and feed intake at ET, since fish typically show either extremely full, or entirely emptied gastrointestinal tracts (Section 1.4.4). It is not obvious whether these inter-individual differences are exclusively due to genetic background, or if ontogenetic ET adaption also accounts for these differences. Since individual adaption to ET starts post-fertilisation (Section 1.6), temperature-differences between hatcheries or hatchery-systems may also be relevant in this context. This may also indicate that the adaption of salmon to ET in hatcheries may be another possible approach to maintain appetence in the summer period.

6.4.2 Replenishment of pigmentation after the resumption of feed intake

Another concept to improve pigmentation quality around the summer period is the replenishment of pigmentation after the resumption of feed intake following temperature extremes and before harvest. Pigmentation of salmon is best achieved by the supplementation of moderate dietary pigment carotenoid concentrations over the entire sea-water grow out phase, rather than high dietary concentrations over short periods (Section 1.3). This circumstance may explain why, to the best of my knowledge, no study tested the carotenoid deposition efficiency of previously unpigmented, large salmon. Due to the pronounced impact of fish size on pigment deposition (Section 1.4.2), it is difficult to estimate how swiftly satisfactory pigmentation in previously unpigmented large salmon (or salmon depleted of pigments) can be achieved. Nevertheless, industry data assessing SalmoFan^{TM}-scores of pre-harvest sized salmon showed that it may take up to six months after resumption of feed intake post summer, to reach similar SalmoFan^{TM}-scores compared to the time before summer (T. Fox-Smith, pers. communication). The fish show extremely high feed intake rates and rapid growth post-summer (T. Fox-Smith, pers. communication), which is likely due to compensatory growth, caused by depleted lipid depots after summer (Section 6.4.1). This may limit the availability of carotenoids for flesh deposition, due to the pronounced, negative correlation of feed intake rates and carotenoid digestibility (Section 1.4.1). This may even be exacerbated due to the rapid growth of muscle tissue during episodes of compensatory growth, since lower amounts of carotenoids are available for flesh deposition (Section 1.4.1) combined with higher amounts of new synthesised white muscle tissue. Hence, restricted feeding may represent a potential tool for streamlined replenishment of pigmentation post-summer, since this approach would improve carotenoid digestibility and reduce white muscle growth. Furthermore, compensatory growth led to increased rates of muscle fibre hypertrophy (Kiessling et al., 1991), which could be indirectly related to muscle

colouration and perhaps pigmentation (Section 6.2). Clearly, further research on the effect of muscle growth and proteolytic processes on pigmentation quality under varying conditions is warranted.

6.4.3 Potential to improve pigmentation quality by the use of recirculation aquaculture systems

The use of large recirculation aquaculture systems (RAS) in salmonid production has vastly increased in recent years, presenting one of the most remarkable changes in production technology, especially in Europe (Bergheim et al., 2009; Dalsgaard et al., 2013). The trend in general is to produce larger salmon smolt to improve performance and resistance in sea net pens. Some producers even plan to conduct the entire production cycle, including the sea water grow-out and finishing phases in RAS. The use of RAS allows the manipulation and control of most water quality parameters inclusive water temperature, which represents a tool to avoid temperature ranges unfavourable for pigmentation in the first place. The manipulation of water temperature is a major cost factor in RAS (Badiola et al., 2012). If the ease of satisfactory pigmentation quality would justify additional electricity cost seems like a rather complex economic question and would depend on the local market, sales strategy and local electricity costs. Anecdotal evidence suggests that salmon swimming in one direction may show differences in the pigment deposition (Skjervold et al., 2001). This could imply that tank reared salmon (e.g. in RAS) may develop inconsistent pigmentation issues. However, one study that tested differences in pigmentation instrumentally and visually (Minolta and salmo fan) found no differences between the fillet half of harvest size Atlantic salmon (Skjervold et al., 2001).

6.4.4. The use of different pigment types and pigment sources

In chapter 2, the loading of salmon with different pigment types (Ax vs. canthaxanthin) did not prevent pigment depletion in a subsequent phase of ET at starvation. It therefore appears that the use of different carotenoid types has little potential to improve pigmentation quality during periods of ET. Nevertheless, due to logistical and financial limitations of the project, only one pigment carotenoid source was used in chapter 3, where the effect of ET on Ax deposition was tested. It can therefore not be excluded that the use of canthaxanthin at the same dietary concentration would have been advantageous for pigment deposition at ET.

As mentioned in Section 1.4.3, there is an increasing demand for natural carotenoids in the pigmentation of salmonids, from sources like e.g. *Phaffia rhodozyma*. It is difficult to estimate whether the use of natural pigments may be beneficial in the pigmentation of salmon at ET. Chemically, the synthetic Ax molecule is identical to synthetic Ax, but differences in the composition of the geometrical (all-E, 9Z and 13Z), and optical (3R,3'R; 3R,3'S and 3S,3'S) isomers between synthetic and natural Ax occur (Bjerkeng et al., 2007; Megdal et al., 2009; Moretti et al., 2006). Hence, if natural Ax sources should contain specific geometrical or optical isomers of Ax that are more stable, or more efficiently deposited in salmon during periods of ET, then the use of natural sources would be of advantage. Salmon fed a synthetic Ax source showed higher amounts of Ix in muscle compared to fish fed diets with the same Ax concentration from a *Phaffia rhodozyma* strain. The metabolic conversion of Ax into Ix in salmon is higher in the Z-isomers (Ytrestøyl and Bjerkeng, 2007). This may explain why higher amounts of Ix were found in the fish fed the synthetic source, which contained higher amounts of these geometrical isomers (Bjerkeng et al., 2007). However, the metabolic conversion of Ax into Ix in muscle was not the underlying mechanism for deteriorated pigmentation at ET (Chapters 3, 4), which makes it seem unlikely that loading salmon with the all-E isomer would be advantageous at ET. Moreover, the differences between Ax from one a strain *Phaffia rhodozyma* and synthetic Ax in the geometrical isomer composition of Ax were rather small (Bjerkeng et al., 2007). Furthermore, there is a significant level of discrimination between different dietary geometrical isomers of Ax for flesh deposition in salmon (Section 1.4.1). This would affect the deposition efficiency of individual geometrical isomers.

Differences in the optical isomer composition can be vast between different natural Ax sources, and the optical isomer composition of Ax in muscle generally reflects the dietary composition (Megdal et al., 2009; Moretti et al., 2006; Østerlie and Bjerkeng, 1999). Hence, salmon muscle could be loaded with certain optical isomers type via dietary manipulation, without deteriorating the utilisation of dietary Ax for muscle deposition. Very little is known about differences in the biological activity of different optical isomers. An *in vitro* study with mice cells unveiled a much stronger immunological response in cells treated with the 3S,3'S isomer of Ax, compared with other optical isomers (Sun et al., 2016). This optical isomer is the predominant isomer found in Ax from *Haematococcus pluvialis*, whereas the synthetic form has a stable optical isomer composition (3S,3'S : 3R,3'S : 3R,3'R; 1: 2 :1) (Moretti et al., 2006; Sun et al., 2016). Future studies could test whether differences in the biological

activities between different optical Ax isomers may have an effect on pigmentation quality in salmon during periods of ET.

6.4.5. Ploidy and transgenic salmon

Tasmania is the only place where triploid salmon are commonly used for production, but there is consideration of using triploids in other countries with naturally occurring Atlantic salmon stocks to prevent interbreeding of escapees from farms with wild salmon (Hansen et al., 2015; Sambraus et al., 2017). Investigations on the effect of ploidy on pigmentation success are scarce, but there is some evidence that triploids may show better pigmentation than diploids held under the same conditions (Bjørnevik et al., 2004). However, triploid salmonids showed lower tolerance, poorer performance and increased mortality compared to diploids at ET (Hansen et al., 2015; Moore et al., 2017; Ojolick et al., 1995 ; Sambraus et al., 2017). One of the major factors that causes reduced pigmentation quality is the cease of feed intake (Section 6.3). Triploid salmon decreased feed intake rates sooner compared to diploids in the course of increasing ambient temperature (Sambraus et al., 2017). It is therefore likely that the increased use of triploids would, alongside higher mortality rates and decreased performance, also lead to deteriorated pigmentation quality in areas exposed to ET. This effect may even be exacerbated by globally decreasing oxygen levels, since mortality and performance of triploids is even more deteriorated, when ET is combined with low (70%) levels of dissolved oxygen (Hansen et al., 2015). Transgenic salmon (here: salmon with growth hormone transgenesis) showed rapid growth towards non-modified salmon and an increased oxygen demand (Cook et al., 2000; Ganga et al., 2015) without an sufficient corresponding adaption in the cardiorespiratory physiology (Deitch et al., 2006). This may be an indication that transgenic salmon may also be more susceptible to ET and decreasing levels of dissolved oxygen. I am not aware of any studies that compared pigmentation quality of size-matched transgenic salmon with non-modified salmon. However, it seems conceivable that the rapid growth of transgenic salmon may lead to similar pigmentation issues compared to non-modified salmon at periods of rapid growth, as seen in Tasmania post-summer (Section 6.4.2). If this should hold true, pigmentation strategies (e.g. restricted feeding, see Section 6.4.2) may be a tool to improve the pigmentation of rapidly growing transgenic salmon.

6.5 Future research

As suggested in Section 6.3, differences in energy metabolism between different fillet areas and temperatures may be involved in pigment depletion at starvation. Energy homeostasis is highly complex and different metabolic pathways are interconnected (Christian et al., 2013; Singh et al., 2009). The apparent upregulation of the peroxosimal β-oxidation may therefore be only one of many changes that may have occurred in areas with pigment depletion. As well as lipids, muscle protein also represents an energy store in teleosts (Carter and Houlihan, 2001). In Chapter 4, despite the pronounced loss in weight and condition during starvation, the difference in chemical composition in muscle tissue before and after starvation was not very pronounced, indicating that muscle protein also served as an energy substrate. It seems very likely that the use of muscle proteins as energy substrate would affect the muscle concentration of pigment carotenoids, since increased protein turnover of muscle also gives more opportunity for Ax to be metabolised (Section 6.2).

Proteolytic processes, therefore processes involved in protein catabolism, can have severe consequences for animals and are therefore tightly regulated (Seiliez et al., 2008). However, the loss of protein from muscle is a physiological feature in anadromous salmonids during spawning migration (Doucett et al., 1999; Seiliez et al., 2012); a life stage at which the fish starve at simultaneously high energy demand, and thereby also mobilise flesh-deposited carotenoids (Choubert and Blanc, 1993; Doucett et al., 1999). Increased proteolytic activity in muscle at this life stage was indicated by increased activity of enzymes of the cathepsin class (Mommsen, 2004; Yamashita and Konagaya, 1990). Enzymes of the cathepsin class were involved in the proteolytic reduction of a range of myofibrillar proteins in isolated white muscle tissue of rainbow trout (*Oncorhynchus mykiss*), including α-actinin (Godiksen et al., 2009). The cathepsin activity in muscle of premature salmonids was affected by temperature (Lerfall et al., 2017) and was increased by stress (Bahuaud et al., 2010) and starvation (Mommsen, 2004) and thus, factors also involved in pigment depletion or the metabolic conversion of carotenoids (see above and Section 1.4.2). Together, these findings may indicate that depletion of muscle-stored carotenoids at starvation is due to increased proteolytic activity. Two main mechanisms as to how pigment depletion at starvation may be caused by proteolytic processes seem thereby conceivable. 1) Changes of structural integrity of myofibrillar proteins and deterioration in their affinityto bind carotenoids; 2) Facilitation of carotenoid depletion by processes involved in muscle proteolysis, without affecting the actual carotenoid binding affinity of myofibrillar proteins.

Hypothetically, both scenarios likely lead to the depletion of the carotenoid pool due to higher opportunity of circulating pigment carotenoids to be metabolised (Section 6.2). At starvation and hence, the cease of dietary carotenoid supply, metabolised carotenoids are not replaced, which would lead to a swift depletion of the carotenoid-pool.

Proteolysis in vertebrates is complex and three major systems are involved; the lysosomal pathway which involves the above-mentioned cathepsin-enzymes, the calpain proteases located in the cytosol, and the ubiquitin-proteasome pathway (Seiliez et al., 2008). Autophagy is the mechanism by which cytosolic components are transported to and catabolised in lysosomes and is considered the most important mechanism in the mass regulation and function of mammalian muscle (Sandri, 2011; Seiliez et al., 2010). Recent studies showed that autophagy is also one important mechanism in proteolysis in rainbow trout muscle at starvation (Seiliez et al., 2010). Autophagy may be of particular importance, since it can be selective for mitochondria and peroxisomes, which may have been involved in the mediation of the switch in lipid metabolism in Chapter 4. The ubiquitin-proteasome dependent proteolysis pathway in rainbow trout muscle may even be reduced in starving rainbow trout (Martin et al., 2002; Seiliez et al., 2008). Differences in the expression of cathepsins and proteasome β, but not calpains in muscle of salmon starved at different temperatures were reported (Carter et al., 2008), demonstrating that the three major proteolytic pathways respond differently to temperature at starvation. Nevertheless, information on the effects of starvation, temperature and other factors on the major proteolytic pathways in salmonid muscle is limited. Even less information is available on the effects of different proteolytic pathways on myofibrillar integrity in muscle *in vivo* and ultimately, the affinity of muscle to bind carotenoids.

Proteolytic processes are likely to affect carotenoid deposition in feeding salmon (Section 6.2) and pigment depletion in starving salmon respectively, and temperature may have a strong impact. In order to examine if and how proteolytic processes affect the pigmentation quality, future research could explore the following objectives:

- Identification of the relative contribution of different proteolytic pathways in changes of myofibrillar proteins in white muscle of fed and starved salmon

- Elucidation as to how the activity of these pathways affect carotenoid depletion, and/or the carotenoid-binding affinity of muscle and how white muscle of different fillet areas is affected at different temperatures

Further, in order to test the potential strategies to improve pigmentation quality at elevated temperature (Section 6.4), the following objectives could be examined:

- Identification of minimal feed intake rates at different temperatures that need to be maintained to prevent pigment depletion (Section 6.4.1)

- Test the potential of different strategies to prevent the cessation of feed intake (Section 6.4.1):
  - manipulation of nutritional status/body condition
  - identification of ingredients/nutrients that promote feed intake at elevated temperature
  - selective breeding and ontogenetic adaption to elevated temperature

- Test the potential to streamline the replenishment of pigmentation following the resumption of feed intake by the means of restricted feeding (Section 6.4.2)

6.6 References

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